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HORMONAL EFFECTS ON THE NUCLEIC ACID AND PHOSPHOLIPID TURNOVER OF RAT LIVER AND THYMUS[1]

J. FRAENKEL-CONRAT[2] AND CHOH HAO LI

From the Institute of Experimental Biology, University of California

BERKELEY

WITHIN the last decade pioneer work by Scandinavian investigators (Caspersson, 1940; Caspersson and Schultz, 1940; Euler and Hevesy, 1942; 1944; Andreasen and Ottensen, 1944; 1945; and Hevesy, 1946) has correlated the turnover of nucleic acids with the rate of growth of certain tissues. Similar findings have also been reported by Brues and his coworkers (1942; 1944), and by Marshak *et al.* (1941; 1945), working with isolated nuclei. The work of Claude (1943; 1944) has also shown the close connection with growth of nucleic acids and phospholipids in several relatively separate components of cytoplasm.

It is well known that hyperthyroid animals possess significantly heavier livers than do normal ones, that the administration of adrenal steroids and of pituitary adrenocorticotropic hormone causes a marked decrease in thymus weight, and that hypophysectomized animals do not gain weight whereas the same animals gain more than normal growing animals if treated with growth hormone. In view of the above findings, experiments were undertaken to ascertain the effects of the thyroid and adrenal hormones, and of pure growth hormone on the turnover of nucleic acids and phospholipids in the liver and thymus of rats.

EXPERIMENTAL

Animals

Group I) Approximately 200 g. female rats which had received stock diet containing 1% desiccated thyroid powder (Armour) for one month and untreated controls.

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[2] Present address: Department of Home Economics, University of California.

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TABLE 1. EFFECT OF VARIOUS HORMONES ON THE NUCLEIC ACID AND PHOSPHOLIPID TURNOVER IN LIVER AND THYMUS OF RATS

Group	Treatment	Blood inorg. P S. A.	Liver nucleic acids		Thymus nucleic acids		Liver phospholipids		Thymus phospholipids	
			S. A.	S. A.	S. A.	R. A.	S. A.	R. A.	S A.	R. A.
I	Normal (2)*	0.96	0.082	0.085	0.106 (1)	0.099† (1)	0.46	0.47	0.134	0.125† (1)
	Thyroid Powder (2)	0.46 (−52%)	0.049 (−40%)	0.106 (+25%)	0.055 (−48%) (1)	0.138† (+39%) (1)	0.13 (−22%)	0.83 (+77%)	0.023 (−83%)	0.057† (−54%) (1)
II	Normal (4)	1.27	0.09	0.071	0.092	0.072	0.61	0.48	0.052	0.041
	17-dehydrocorticosterone (2)	1.48 (+17%)	0.10 (+11%)	0.068 (−4%)	0.116 (+26%)	0.079 (+10%)	0.45 (−26%)	0.30 (−38%)	0.070 (+36%)	0.047 (+15%)
	Cortical Extract (2)	1.12 (−11%)	0.10 (+11%)	0.089 (+25%)	0.111 (+21%)	0.099 (+38%)	0.61	0.54 (+13%)	0.051 (−2%)	0.046 (+12%)
III	Normal (4)	1.51 (3)	0.15	0.99	0.110 (3)	0.073	0.67	0.44	0.062 (3)	0.041 (3)
	Hypophysectomy (3)	2.98 (+97%) (2)	0.13 (−13%)	0.44 (−56%)	0.069 (−37%) (2)	0.023 (−68%) (2)	0.38 (−43%)	0.13 (−71%)	0.027 (−56%) (2)	0.009 (−78%) (2)
	Growth Hormone in Hypophysectomized Rats (3)‡	1.94 (−35%) (2)	0.14 (+8%)	0.72 (+64%)	0.108 (+57%) (2)	0.056 (+144%)	0.55 (+45%)	0.28 (+115%)	0.063 (+133%) (2)	0.033 (+267%) (2)

* Figures in parentheses indicate number of rats; duplicate analyses were made of liver fractions, single analyses of thymus fractions.
† Based on R. A. of blood being 1.07 and 0.40 for normal and hyperthyroid animals respectively.
‡ Percentage differences expressed for values obtained with tissue from growth hormone treated hypophysectomized rats are based on values obtained with untreated hypophysectomized controls.

Group II) Approximately 90 g. female rats which had received a daily subcutaneous injection of a total of (a) 18 mg. 17-dehydrocorticosterone (Merck) over a period of 8 days or (b) 25 R. U. Cortical Extract (Upjohn) over a period of 11 days, and (c) untreated controls.

Group III) Approximately 75 g. female hypophysectomized rats which had received daily intraperitoneal injections of 0.170 mg. pure growth hormone per day for 10 days and untreated hypophysectomized as well as normal controls.

Material

Radioactive phosphorus (P^{32})[3] was administered intraperitoneally as inorganic phosphate on an average of 1.5 μc. per 100 g. rat, 2 hours prior to sacrifice.

Procedure

The animals were anesthetized with ether; a blood sample was taken from the inferior vena cava for determination of the specific activity of free plasma phosphate; the liver and thymus were removed, weighed, and promptly immersed in cold saline (0°C.). Tissue samples were weighed on a Roller-Smith torsion balance, homogenized in cold 4% trichloroacetic acid and separated into phospholipid, nucleic acid, and residual protein phosphorus fractions according to the method of Schneider (1945). Duplicate samples were analyzed for each liver; whereas the whole thymus was required for each analysis. Radioactivity was determined with the Geiger-Müller counter and chemical determination of phosphorus done by the Sumner method (1944).

RESULTS AND DISCUSSION

The results are given in terms of specific activity (S. A.) and relative activity (R. A.). Specific activity is defined as the ratio of the percentage of administered P^{32} to mg. $P.^{31}$ The relative activity is defined as the ratio of the specific activity of the fraction in question to the specific activity of the free plasma phosphate. The results are summarized in Table 1.

In the present studies it has been found that the complete cessation of growth as brought about by hypophysectomy in a young animal, results in a relative decrease in the turnover of nucleic acids and phospholipids as compared with unoperated animals. Conversely, treatment with growth hormone has caused a suggestive increased turnover of these fractions. Significant responses were elicited in the thymus, which increased 74% in weight, compared with its hypophysectomized control; whereas the responses in the liver, which increased by only 30%, were not as great.

Smaller and less consistent effects were obtained in the studies conducted with other hormone. Hyperthyroidism which caused enlargement of the liver (69% increase) led to an increased turnover of

[3] Kindly supplied by the Radiation Laboratory, University of California, Berkeley.

nucleic acids and phopholipids of the liver and of thymus nucleic acids but a decrease in the thymus phospholipid turnover.[4]

Treatment with 17-dehydrocorticosterone, which occasioned a slight loss of body weight (6 g.) and more considerable loss in thymus weight (32%) and liver weight (18%), did not cause the anticipated decrease in either nucleic acid or phospholipid turnover. Perhaps if larger doses of this steroid had been administered, more marked responses might have been obtained.

Cortical Extract in contrast to the cortical steroid, caused an increase in body weight (45 g.) and in liver weight (36%), and only an insignificant decrease in thymus weight (12%). In the case of both liver and thymus a slight increase of nucleic acid and phospholipid turnover occurred.

Studies in which only total nucleic acids are estimated might seem of questionable value since desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have such diverse functions. In these preliminary studies however we have been able to show clear effects especially in the thymus of hypophysectomized animals with and without growth hormone. All of these results on total nucleic acid turnover are undoubtedly measurements primarily of RNA, since it has been shown that there is about four times as much RNA as DNA in tissues (Brues, Tracy and Cohn, 1944; Davidson and Waymouth, 1943), and that the turnover of RNA is always greater than that of DNA (Hammarsten and Hevesy, 1946).

SUMMARY

Hypophysectomy was associated with a decreased turnover of both nucleic acids and phospholipids in the rat's thymus and of the phospholipids in rat liver, with less effect on liver nucleic acids.

Growth hormone administered to hypophysectomized animals caused an increased turnover of nucleic acids in the thymus and of phospholipids in both liver and thymus.

Thyroid treatment was accompanied by a decreased specific activity of both nucleic acids and phospholipids in both liver and thymus, an increased relative activity of the phospholipids of the liver but decreased relative activity of the phospholipids of the thymus. It caused an increased relative activity of the nucleic acids in both organs.

Both 17-dehydrocorticosterone and Cortical Extract led only to slight changes in the turnover of either substance in both organs.

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[4] The great difference in magnitude of the values for the S. A. and R. A. of the nucleic acids and phospholipids observed in the hyperthyroid animals was in agreement with similar differences observed in the S. A. and R. A. of adenosinetriphosphate values observed in previous experiments (D. M. Greenberg, J. Fraenkel-Conrat and M. B. Glendening, unpublished). There difference has been attributed to the dilution of injected P^{32} by a great increase in plasma inorganic P^{31}.

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A MODIFIED PETTENKOFER REACTION FOR THE QUANTITATIVE ESTIMATION OF DEHYDROISOANDROSTERONE AND ITS APPLICATION TO ANALYSIS OF URINARY EXTRACTS AND FRACTIONATIONS[1,2]

LORENZ HANSEN

From the Jefferson Medical College, Department of Biochemistry

PHILADELPHIA, PENNSYLVANIA

WHILE the author was engaged in developing a colorimetric method specific for dehydroisoandrosterone (DA) (Hansen, 1948) a report by Kerr and Hoehn (1944) on the application of a modified Pettenkofer reaction for the qualitative detection of various steroids came in hand. They found DA to give a positive reaction and suggested that the method might, perhaps, be applied to the quantitative estimation of this substance. The procedure as employed by Kerr and Hoehn was applied to qualitative and quantitative comparison of available steroids, and more specifically to DA in pure solutions, in various urinary extracts, and in such extracts with addition of known quantities of DA. In order to apply the principle of this method more satisfactorily, it seemed desirable to modify the procedure in various respects: to increase and stabilize the color; to simplify the technic; and to further test for the specificity and suitability for quantitative estimation of DA. Experiments performed with these objectives in view are described in this paper.

EXPERIMENTAL RESULTS

Factors applied to enhance and stabilize the color and simplification of technic: it was decided to make whatever changes might be necessary to permit the use of a boiling water bath instead of specially controlled temperature baths. Since the color production involves (a) reaction of the steroid with furfural, and (b) subsequent color development in an acid solution, modifications were adopted for favorable results (1) with reference to change under (a) and (2) sub-

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[2] A preliminary report was presented at the Meeting in Miniature of the Philadelphia Section of the American Chemical Society in Philadelphia on January 20, 1949.

sequently further changes for maximum improvement with reference to color development under (b).

Choice of heating and time in the water bath: Four different series of tests were done, the general procedure being as follows: To the evaporated and dried alcoholic aliquot in a test tube, 3.5 cc. of 60% acetic acid (by volume) was added and brought to the temperature of the water bath, then 1 cc. of 0.9% furfural (redistilled furfural, 0.9 cc. per 100 cc. of aqueous solution) was added, mixed, and then heated in the bath as indicated in each of the series under (a) in Table 1. This was followed by addition of 6 cc. of 16 N H_2SO_4, mixed, and heated in the bath as indicated in Table 1 under (b). Finally, 2.5 cc. of acetic acid was added to each tube, mixed, the tube was cooled in an ice bath, and colorimetry was done in an Evelyn

TABLE 1. CHOICE OF HEATING TIME FOR FAVORABLE COLOR DEVELOPMENT

DA* μg.	Series			
	1	2	3	4
	(a) 10 Min., 70°C. (b) 10 Min., 74°C.	(a) 10 Min., b. H_2O B.* (b) 10 Min., 74°C.	(a) 10 Min., b. H_2O B. (b) 10 Min., b. H_2O B.	(a) 5 Min., b. H_2O B. (b) 5 Min., b. H_2O B.
	% T	% T	% T	% T
25	81[1] (83)	90[2] (90[1])	86 (85)	85 (84)
50	76 (76[1])	77 (77[2])	73 (73)	71 (71[2])
75	69 (70)	68 (68)	66 (66)	60 (60[2])
100	63[3] (65)	62[3] (64)	55[2] (56)	52 (53)

* DA = dehydroisoandrosterone; b. H_2O = boiling water bath; T = % transmission with filter 660 in an Evelyn colorimeter. The first column under %T indicates the first reading, the column in ( ) gives values an hour later.

photoelectric colorimeter with filter 660, which uniformly gave highest absorption values. The % transmission was recorded and again an hour later. Table 1 indicates the results obtained with 4 different aliquots of DA. Thus, the data indicate that (a) the boiling water bath may be substituted in place of a special temperature bath, (b) color produced is stable for at least an hour, (c) heating time can be reduced in the boiling water bath to 5 minutes for each of the 2 reactions with favorable enhancement in color intensity and without loss in stability.

Choice of concentration of acid and heating time in acid solution: The object was to further improve color development by modifying the sulfuric acid and the time period in the second heating. The first part was carried out as previously indicated, employing 5 minutes heating in a boiling water bath. The second heating period was modified to employ 6 cc. of varying concentrations of sulfuric acid, heating the more favorable concentrations for varying time periods, cooling in an ice bath, omitting the final addition of 60% acetic acid, and reading with an Evelyn colorimeter soon after dilution and again

an hour later. Data in Table 2 indicate results obtained. Conclusions drawn from these data may be summarized as follows: (a) with increase in concentration of sulfuric acid the color also increases, but there is also a parallel color increase in the respective reagent blanks; (b) to avoid a too deeply colored blank 19 N H_2SO_4 appears to be favorable for nearly maximum blue color for DA; (c) heating for 3 minutes is the most favorable time period, less than that produces much less color with DA and more does not greatly increase the blue

TABLE 2. CHOICE OF CONCENTRATION OF SULFURIC ACID AND HEATING TIME AFTER ADDITION OF THE ACID

H_2SO_4 N conc.	Heating in min.	25 μg DA* % T*	50 μg DA % T	75 μg DA % T	100 μg DA % T	Blank % T
13.22	3 (7)	94[3]	90	85	81[2]	100
16.00	3	93 (92)	85[1] (83)	80 (77)	72[3] (69)	98
16.85	3	83 (82)	70 (68[2])	60[1] (58[2])	51[1] (49[2])	96
17.83	2	83 (82[1])	72 (71)	65[2] (63[1])	60 (58)	97[2]
17.83	3	79 (79)	64 (63)	51 (50)	43 (42)	92
17.83	4	76[1] (76)	61 (61)	49 (49)	42 (42)	93[1]
17.83	5	77 (78)	62 (63)	49 (49)	40 (40)	89
18.85	2	76 (75[3])	61 (61)	50 (49[2])	42 (41[2])	95[2]
18.85	3	71 (72)	52 (53)	41 (42)	33 (33[2])	90[2]
18.85	4	75[2] (76)	53 (54)	43 (44)	35 (35[2])	86
18.85	5	73 (74[1])	54 (55[3])	41[2] (43[1])	34 (35[2])	82[3]
19.89	3	70 (71)	56 (57)	39[2] (40)	31[2] (32)	87
20.94	3	65[3] (66)		36[2] (37)	28[1] (28[3])	86[2], 88
21.67	3	68 (69)	51 (51[2])	37[2] (38)	29[3] (30)	79[2]
23.14	3	65 (65)	47 (47)	33 (33)	27 (27)	77

* DA = dehydroisoandrosterone; T = % transmission in an Evelyn colorimeter with filter 660. Columns under % T are first reading and in ( ) readings after an hour. The readings are all with the reagent blank, with sulfuric acid of the concentration for a given horizontal column, set at 100. The last column is a comparison of the reagent blanks against a reagent blank with 13.22 N H_2SO_4 set at 100.

color, while the color of the blank is appreciably deepened. In subsequent series of tests employing 19 N H_2SO_4 and read against blanks without furfural set at 100, the reagent blank readings varied between extremes of 88 and 93 with an average of 90 for 10 seperate determinations.

Method adopted for quantitative determination of DA: An alcoholic aliquot in a test tube containing DA within the range of 10–100 μg. is evaporated in a hot water bath and dried over $CaCl_2$ in a vacuum desiccator. To each tube, including blanks, 3.5 cc. of 60% acetic is added, which is then heated in a boiling water bath to bring the tube to the temperature of the bath, then add 1 cc. of 0.9% furfural solution, mix, heat in the boiling water bath for 5 minutes. Then add 6 cc. of 19 N H_2SO_4, mix, and heat for 3 minutes in the boiling water bath. Cool at once in an ice bath. Transfer to Evelyn tubes and read in an Evelyn colorimeter with filter 660 in 30 to 60 minutes after the final heating.

No special reagents are required except redistilled furfural. Fur-

fural solution was prepared fresh on the day it was used. However, furfural solutions kept refrigerated for a number of days showed no greater variations with different aliqouts of DA than did similar aliquots with freshly prepared furfural solutions. Baker's C.P. glacial acetic acid was employed for preparation of 60% acetic acid (60 cc. of glacial acetic and 40 cc. of distilled water). Baker's C.P. H_2SO_4 was used to prepare 19 N solution of the acid (50–50 by volume with distilled water, with subsequent adjustment to make the solution 19 N within 0.1N) .

Spectrophotometric adsorption curve: The adsorption curve of the color produced with DA by the indicated procedure was determined with a Beckmann spectrophotometer over the range of 325 to 900 mμ. With density plotted as ordinate for the mean of 2 determinations with 100 μg. DA, against the wave length in mμ. employed as abscissa, 2 maxima occur, one at 656 mμ. and the other, much lower value, at 370 mμ. There is also a slight plateau on the ascending curve from 610–620 mμ. Thus, photometrically the highest color value is obtained at 656 mμ., which confirms the optimum value obtained with filter 660 in the Evelyn colorimeter.

In subsequent data the 660 filter was employed with the Evelyn colorimeter and evaluations were made on the basis of the values of standard solutions of DA determined along with unknowns. All standards and unknowns were made up in absolute alcohol solution and suitable aliquots were evaporated to dryness in a hot water bath and dried further over $CaCl_2$ in vacuo, followed by the procedure indicated above.

Method of calculation of DA in specimens: Calculations were made on the basis of the mean color density per μg. of DA. A reagent blank served as control in case of pure solutions. In case of aliquot of unknowns a specimen blank and a reagent blank were obtained against a zero blank, i.e., a blank containing water in place of furfural, to make proper allowance for the non-specific colors in the unknown and in the reagent blank. Thus, for the determination of DA in an unknown, the following set of tubes is employed: (1) zero blank, reagents with water in place of furfural, read ultimately at 100 with filter 660 in the Evelyn Colorimeter; (2) reagent blank, for non specific color value of reagents with furfural; (3) a series of tubes with DA within the limits of 10–75 μg.; (4) an aliquot of unknown without furfural, for the non specific color in the unknown; and (5) an aliquot of unknown, as in tube (4), with furfural. The net color density for the standard DA tubes is obtained by subtraction of the value in (2) from that in each of the tubes in (3). Dividing the result by the μg. employed gives the density per μg. of DA. The mean value for the series of standards is then calculated. The net color density value for the unknown is obtained by subtracting the sum of densities in (2) and (4) from the value in (5). Dividing this value by the

mean color density per μg. of DA then gives the μg. of DA in the aliquot of the unknown. Since the specimen blank is usually small, procedure and calculations may be simplified in most cases by omitting the zero blank and the specimen blank and setting the reagent blank at 100 on the galvanometer scale. This introduces but a small error in most cases.

Table 3 presents data obtained with many determinations of aliquots of DA ranging from 10–150 μg. The weighted mean density value is calculated in accordance with the density and number of determinations employed for each of the various aliquots. A curve plotted on the basis of the calculated density values for the range 10–100 μg. DA (column 4, Table 3) and employed for placing mean

TABLE 3. DEHYDROISOANDROSTERONE (DA) DENSITY VALUES OF RANGE 10–150 μg.

DA μg.	Observed density mean value	Density per μg. $\times 10^5$	Density calculated
10	0.0530 (3)	530	0.0492
15	0.0744 (4)	496	0.0738
20	0.1001 (3)	500.5	0.0984
25	0.1288 (12)	515	0.1231
50	0.2480 (11)	496	0.2461
65	0.3470 (1)	533.8	0.3199
75	0.3584 (9)	477.9	0.3692
90	0.4690 (1)	421.1	0.4430
100	0.4580 (12)	458	0.4922
125	0.5270 (3)	421.4	—
150	0.6580 (1)	438.7	—

Weighted mean density 492.2 (10–100 μg.)

The number in ( ) in the second column indicates the number of determinations employed. Density $= 2 - \log \cdot G$, where G is the galvanometer reading with the reagent blank set at 100 and read with filter 660 in an Evelyn colorimeter.

values of individual determinations (column 2, Table 3) and other single determinations of DA indicated that points for 10 to about 70 μg. of DA lie close to the straight line curve, but points for values above this range deviate more and more and fall below the line. Thus, the color is reasonably valid for measurements in the range of 10–70 μg. of DA. However, since small variations do occur, even though all conditions are strictly adhered to, a set of standards has been used with all determinations, instead of relying in a general calibration curve.

Determination of DA in presence of androsterone: Solutions of mixtures of these 2 steroids in various ratios and absolute amounts were estimated for DA values by the procedure as indicated. Table 4 indicates the results obtained. It may be noted that the values tend to be low when large aliquots are mixed with androsterone, but large aliquots should be avoided in any case.

Specificity of the reaction with steroids of various types: A comparison of 100 μg. aliquots of substances which produced a positive color is indicated in Table 5. All of the steroids tested may be con-

TABLE 4. DETERMINATION OF DEHYDROISOANDROSTERONE (DA) IN THE PRESENCE OF ANDROSTERONE (A)

DA μg.	A μg.	Ratio DA/A	Observed density*	Observed density
				Density of DA
25			0.1308	1.00
25	37.5	0.67	0.1367	1.04
25	50	0.5	0.1235	0.945
25	100	0.25	0.1295	0.99
50			0.2347	1.00
50	25	2.0	0.2339	0.995
50	50	1.0	0.2366	1.01
50	75	0.67	0.2518	1.07
50	100	0.5	0.2666	1.13
100			0.4800	1.00
100	25	4.0	0.4395	0.915
100	50	2.0	0.4545	0.945
100	100	1.0	0.4255	0.885

* Density $= 2 - \log \cdot G$, where G is the reading of the galvanometer. All readings were made with the reagent blank set at 100 with filter 660 in an Evelyn colorimeter.

veniently grouped according to general structure and carbon content. These substances were all tested in 100 μg. aliquots, and in some instances in amounts up to 1 and 5 mg. Except where followed by a (+) they were all similar to the reagent blank. Androstane type, C19: androsterone, isoandrosterone (+), androstanediol-3β, 17α, androstenediol-Δ^5-3β, 17α (+), 17-methyl-androstanediol-3β, 17α (+), 17-methyl-Δ^5-3β, 17α -androstanediol (+), 17-ethyl-Δ^5-3β, 17α -androstenediol (+), androstenedione, dehydroisoandrosterone (+), dehydroisoandrosterone acetate (color value equal to the steroid content unesterified), testosterone, cis-testosterone (+), testosterone propionate, methyl testosterone, ethinyl testosterone. Estrane type, C_{18}: estrone, α-estradiol, α-estradiol benzoate, α-estradiol dipropionate, ethinyl-α-estradiol. C_{21} type: progesterone, desoxycorticosterone acetate, pregnenolone (+), C_{24} type, bile acids: cholic acid (+), desoxycholic acid, lithocholic acid, 3, 12-dihydroxy-7-ketocholanic

TABLE 5. POSITIVE COLOR AND DENSITY OBTAINED WITH VARIOUS STEROIDS

Substance	Color	Color density*
Cholic acid	blue	0.2596
Dehydroisoandrosterone	blue	0.4140
Androstenediol-Δ^5,3β,17α	blue, purplish tint	0.4090
Pregnenolone	blue with green tint	0.2366
Isoandrosterone	faintly blue	0.0593
Cis-testosterone	blue, dark hue	0.0809
17-methyl-Δ^5-androstenediol-3β,17α	purplish tint	0.0731
17-ethyl-Δ^5-androstenediol-3β,17α	purplish-pink	0.1065
17-methyl-androstanediol-3β,17α	faintly blue	0.0302

* Density $= 2 - \log \cdot G$, where G is the galvanometer reading. All density values are the mean of 2 determinations with 100 μg. of substance. Readings were made with the reagent blank set at 100 and with filter 660.

TABLE 6. ESTIMATION OF DEHYDROISOANDROSTERONE IN A VARIETY OF URINARY EXTRACTS

Specimen	Aliquot used cc.	DA found in aliquot μg.	Da found* per cc. mg.	Da found in total specimen mg.
β Fraction, pooled, volume 10 cc.	0.10	33.20	0.332	
	0.15	47.41	0.316	
5β, volume 5 cc., dilution 1-3	0.05	36.59	2.195	10.977
	0.10	72.69	2.181	10.904
B-1126, before fractionation, vol. 10 cc. diln. 1-10	0.10	10.21	1.021	10.210
	0.20	20.97	1.049	10.485
S-, pooled β fractions, dilution 1-25	0.10	31.84	7.96	
	0.20	70.08	8.76	
B-749, before fractionation, vol. 10 cc., diln. 1-10	0.05	59.29	11.858	118.58
	0.10	102.9	10.29	102.9

* DA = dehydroisoandrosterone, calculated on the basis of a zero blank and with a set of pure DA solutions as standards.

acid, 3, 7-dihydroxy-12-ketocholanic acid, 3, 6-diketo cholanic acid, 3, 12-diketo cholanic acid, 3-keto-6-hydroxy-cholanic acid, 3, 7-diketo cholanic acid, 3-hydroxy-7, 12-diketo cholanic acid. C_{27}: cholesterol. . . . Some of the substances other than those indicated by (+) produce a slight yellow color when aliquots above 500 μg. and up to 2 mg. are employed. In case of cholesterol with 1 and 2 mg. aliquots the % transmission was 96 and 94, respectively, with filter 620.

Estimation of DA in urinary extracts: Examples are presented in

TABLE 7. RECOVERY OF DA ADDED TO VARIOUS URINARY EXTRACTS

Specimen*	Aliquot used cc.	DA added μg.	DA found μg.	DA recovered	
				μg.	%
Before fractionation					
B-1126, 10 cc.	0.20		20.97		
dilution 1-10	0.20	25	46.38	25.41	101.6
B-1149, 10 cc.	0.20		25.37		
dilution 1-10	0.20	25	49.74	24.37	97.5
Ketone fractions					
B-569, 5 cc.	0.10		40.57		
dilution 1-25	0.10	25	66.48	25.91	103.6
E-, pooled	0.10		19.61		
(deep red color)	0.10	25	43.08	23.47	93.9
β fractions					
11β, 5 cc.	0.10		69.21		
dilution 1-3	0.10	25	93.99	24.78	99.1
17β, 5 cc.	0.10		55.63		
dilution 1-3	0.10	25	82.79	27.16	108.6

* The specimens are urine extracts from adrenal tumor cases. DA = dehydroisoandrosterone.

Table 6. The values obtained with different aliquots check quite well in the first 3 examples, but in the last 2 examples they are not so satisfactory. In the last example, however, the lower value was to be expected on the basis of the high content in the larger aliquot.

Recovery of DA added to urinary extracts: Data are presented in Table 7. Two examples are given for each of 3 types of extracts, (a) neutral steroids before fractionation, (b) ketone fractions of neutral steroids, (c) beta fractions of digitonin fractionations of ketone fractions. The specimens employed are urine extracts from adrenal tumor cases.

TABLE 8. COMPARISON OF VARIOUS METHODS FOR THE DETERMINATION OF DEHYDROISOANDROSTERONE IN β FRACTIONS

Method†	Specimens*					
	A mg. DA	B mg. DA	C mg. DA	D mg. DA	E mg. DA	F mg. DA
1	11.289	12.238	12.565	12.421	7.913	9.404
2	11.325	12.390	12.0	12.0	11.325	12.15
3	10.70	11.140	10.615	11.35	7.465	9.20
4	11.232	10.997	12.192	11.853	8.35	9.408

* The specimens represent pairs of digitonin fractionations of ketone fractions from urinary extracts of 3 adrenal tumor cases. The duplicate pairs are A and B, C and D, and E and F, each represents $\frac{1}{4}$ of the total ketone fraction of a 24 hr. urine extract. A part of specimen E was lost in the manipulation.

† Method, Holthorff and Koch's adaptation (1940) of Zimmermann's method for ketosteroids (1935) slightly modified by the author (1943); method 2 is the method of Pincus (1943) employing $SbCl_3$ as the color reagent; method 3 is specific for dehydroisoandrosterone, developed by the author (1948); method 4 is the present method.

Comparison of various methods for the determination of DA in beta fractions: Results are indicated in Table 8 for DA determined in urinary extracts by 4 different methods. Only beta fractions could be employed, since the other methods could be applied only to such fractions for direct comparison.

DISCUSSION

The data here presented have indicated that estimation of pure DA can be done with reasonable precision by this modified Pettenkofer reaction. Further, the presence of androsterone does not interfere appreciably in this estimation within the working range of the method. In applying the procedure to urinary extracts added DA is nearly completely recovered from such extracts, whether they be unfractionated, ketone fraction or β fraction. Finally, comparison of determination of DA in fractions of urinary extracts by this procedure with several other methods indicates reasonably close agreement. The method may, therefore, be employed for reasonably precise determinations of DA in urinary fractions. It may also be used in preliminary analyses of unfractionated and ketone fractionated ex-

tracts. Munson *et al.*[3] (1948) have developed the analysis for DA, based on the Pettenkofer reaction. Results obtained by the method here presented appear to be similar qualitatively and quantitatively, but this method has the advantage of being simpler in technic.

While analyses for DA in pure solutions and in various urinary extracts seem reasonably satisfactory, comparison with other steroids indicates that the specificity is far from being unique for DA. The data presented here on specificity is in substantial agreement with the interpretations of Munson *et al.* (1948) and with the data of Kerr and Hoehn (1944). Certain structural features of the steroid nucleus appear to be required for a positive reaction. The most important features appear to be Δ^5 unsaturation, along with color enhancing, β-directed -OH (or other univalent group) in C_3 position. Good examples are DA; Δ^5-androstenediol-3β, 17α; Δ^5-pregnenol-3β-one-20; and 3-hydroxy-Δ^5-cholenic acid (lithocholenic acid). Saturation on C_5, as in isoandrosterone, greatly reduces the color, while a simultaneous saturation on C_5 and reversal of the -OH on C_3, as in androsterone, abolishes the color completely. Retention of unsaturation on C_5 and removal of the functional group on C_3, as in $\Delta^{4,5}$-androstanedione-17, reduces the color only in a small degree. However, certain anomalies appear. Thus, cholesterol, which has the above specified specific conditions gives no color, and cholic acid, in which the specific conditions are absent gives the color. Substitution of hydrogen on C_{17} is also of some significance, since alkyl substitution greatly diminishes the chromogenicity. Oxidation of secondary alcohol groups in cholic acid and chenodesoxycholic acid to the corresponding dehydro acids also abolishes the color, indicating importance of structural features of the steroid nucleus other than on Carbons 3, 5 and 17.

It was hoped that this colorimetric method might be sufficiently specific so that β fractionation or even ketone fractionation might be unnecessary. This hope, however, is not realized, for α fractions of specimens hitherto tested all contained more or less chromogenic substance qualitatively the same as for DA, and in some unfractionated urine extracts the color equivalent, calculated as DA, represented a larger amount of DA for the total specimen than was found by the Zimmermann technic in the same specimens calculated as androsterone (A). Obviously, in the unfractionated specimen substances other than DA or A are indicated by this procedure. Likewise in the α fraction, hence also in the ketone fraction prior to digitonin fractionation, substances other than DA are also indicated. In the β fraction, as has been shown, determination of DA in various specimens by comparative methods indicated nearly identical results by

[3] This report first came to the author's attention when the work here presented was virtually completed.

4 different methods. Nevertheless, even here substances similar to DA chromogenically cannot be entirely excluded.

Finally, these various specificity aspects may be of use in indiacting or excluding the presence of substances which possess structural features chromogenically favorable in this reaction.

SUMMARY

A colorimetric method has been developed for the quantitative estimation of dehydroisoandrosterone, applicable to pure solutions and to urinary neutral steroid extracts.

The method is based on a modified Pettenkofer technic, employing a boiling water bath as source of heating, an acetic acid solution with addition of furfural for the initial reaction, and addition of sulfuric acid for the final color development.

Specificity is discussed on the basis of tests on a variety of different types of steroids as reported here and as indicated in reports elsewhere. Specificity is not unique for dehydroisoandrosterone. In general, it is indicative of β OH on C_3 and unsaturation on C_5, but is not conclusive.

Application of the method to quantitative estimation of dehydroisoandrosterone in fractions of urinary extracts is reasonably satisfactory. Limitations of the application to unfractionated specimens or ketone fractions are discussed. Use of the method to indicate presence or absence of substances with favorable chromogenic structural features is suggested.

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THE RELATIVE GROWTH OF THE THYROID GLAND IN THE BOVINE FETUS[1]

C. W. NICHOLS, JR., I. L. CHAIKOFF, AND J. WOLFF[2]
From the Division of Physiology of the University of California Medical School
BERKELEY

THE NATURE of thyroid iodine in the postnatal state is fairly well understood. At least 90 per cent of it is organic and almost all of this can be accounted for by two fractions: thyroxine-like and diiodotyrosine-like iodine.[3] These two compounds do not exist in the free state in the gland but are combined there with other amino acids to form the characteristic thyroid protein, thyroglobulin.

The nature of the iodine in the fetal organism has not been clearly established. To obtain such information, a study of various iodine fractions of the bovine fetal thyroid was undertaken. Data on that phase of the study will be presented in the following paper. The material acquired during these experiments also provided an opportunity for studying the growth of the gland in relation to body weight, body length, and age of the bovine fetus from 62 days to term (278–285 days depending upon breed). The present paper is concerned with this phase of the study.

EXPERIMENTAL

Intact uteri of pregnant beef were collected randomly at abattoirs at the time of slaughter. The embryos and fetuses were weighed and measured immediately after the umbical cord was tied off at the fetal level. The thyroids were then removed, freed of extraneous connective tissue and fat, and weighed. A small portion was fixed in Bouin's solution for histological examination.

The fetuses were coellcted from random herds. Breeding or stock histories were not available. The present series comprises a total of 121 bovine fetuses obtained from dams chiefly of the Hereford breed (97 specimens). Four fetuses were obtained from Guernsey, 15 from Holstein, and five from Jersey breeds. Since the slopes of curves relating thyroid weight to body weight did not differ for the two sexes, no distinction was made between male and female fetuses.

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[1] Aided by grants from the U. S. Public Health Service and from the Committee on Endocrinology of the National Research Council.
[2] U. S. Public Health Fellow.
[3] Monoiodotyrosine has recently been found in the thyroid gland by Fink *et al.* (1948) and by Taurog *et al.* (1949).

RESULTS

In order to determine fetal ages, we employed the data of Winters *et al.* (1942) who measured body dimensions of fetal calves of known ages. In the present study, the age of each fetus was taken as the average of ages derived from the following four measurements: body weight, crown-rump (C.R.) length, chest circumference, and abdominal circumference. These were determined by methods previously described (Nichols, 1944).

The ages of the fetuses ranged from 62 days to term. In table 1, they have been grouped with respect to arbitrarily selected age ranges and the body measurements have been recorded for each range.

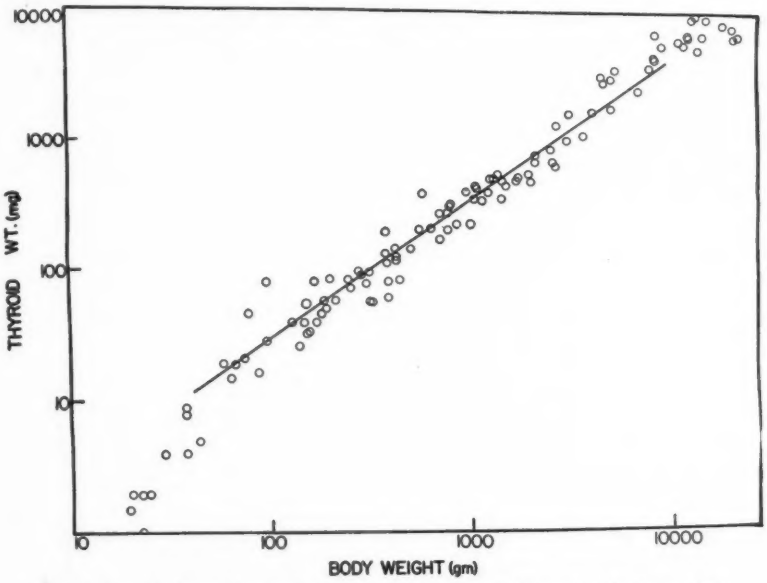

FIG. 1. Double-logarithmic plot of fetal thyroid weight against body weight.

When thyroid weights were plotted on a double logarithmic grid against total body weight (fig. 1), C. R. length (fig. 2), and calculated age (fig. 3), straight-line curves were obtained.

The data shown in figs. 1–3 were therefore fitted to the simple allometry equation of Huxley (1932):

$$y = bx^k \qquad (1)$$

where y = thyroid weight expressed in mg.

x = body weight expressed in gm. (fig. 1), or C.R. length in mm. (fig. 2), or age in days (fig. 3).

TABLE 1. THYROID WEIGHTS AND BODY DIMENSIONS OF FETAL CALVES FROM 62 DAYS TO TERM

Calculated age	No. of fetuses	Body weight		C.R. length		Chest circum.		Abdom. circum.		Thyroid weight	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
days		gm.	gm.	mm.	mm.	mm.	mm.	mm.	mm.	mg.	mg.
62–70	9	18–34	27	73– 96	85	51– 75	66	61– 80	70	1–9	4
75–85	10	42–150	84	104–153	127	87–120	99	82–122	100	5–45	25
87–95	14	96–235	168	151–185	165	115–133	123	115–149	129	24– 82	53
98–105	16	240–433	350	178–222	203	142–168	154	144–174	161	57–183	90
106–120	15	433–860	639	215–273	245	167–208	189	175–214	183	117–348	200
125–135	11	$0.98 \cdot 10^3$–$1.50 \cdot 10^3$	$1.21 \cdot 10^3$	295–334	312	214–253	233	227–265	252	202–471	350
136–150	9	$1.52 \cdot 10^3$–$3.13 \cdot 10^3$	$1.94 \cdot 10^3$	325–418	353	243–352	276	270–354	295	$0.32 \cdot 10^3$–$1.37 \cdot 10^3$	542
152–160	9	$2.10 \cdot 10^3$–$3.82 \cdot 10^3$	$2.76 \cdot 10^3$	382–462	408	280–331	301	282–342	320	$0.42 \cdot 10^3$–$1.10 \cdot 10^3$	709
163–175	7	$4.34 \cdot 10^3$–$5.60 \cdot 10^3$	$4.95 \cdot 10^3$	445–590	511	349–435	381	367–453	394	$1.13 \cdot 10^3$–$2.74 \cdot 10^3$	$2.01 \cdot 10^3$
190–210	9	$7.25 \cdot 10^3$–$13.0 \cdot 10^3$	$9.83 \cdot 10^3$	573–703	622	408–508	461	428–564	486	$2.85 \cdot 10^3$–$6.69 \cdot 10^3$	$4.01 \cdot 10^3$
213–228	5	$12.5 \cdot 10^3$–$15.7 \cdot 10^3$	$14.5 \cdot 10^3$	700–735	710	535–562	548	532–597	575	$3.91 \cdot 10^3$–$6.95 \cdot 10^3$	$5.40 \cdot 10^3$
240–260	7	$19.9 \cdot 10^3$–$32.5 \cdot 10^3$	$24.7 \cdot 10^3$	790–855	838	617–676	650	645–705	673	$4.59 \cdot 10^3$–$11.4 \cdot 10^3$	$6.54 \cdot 10^3$

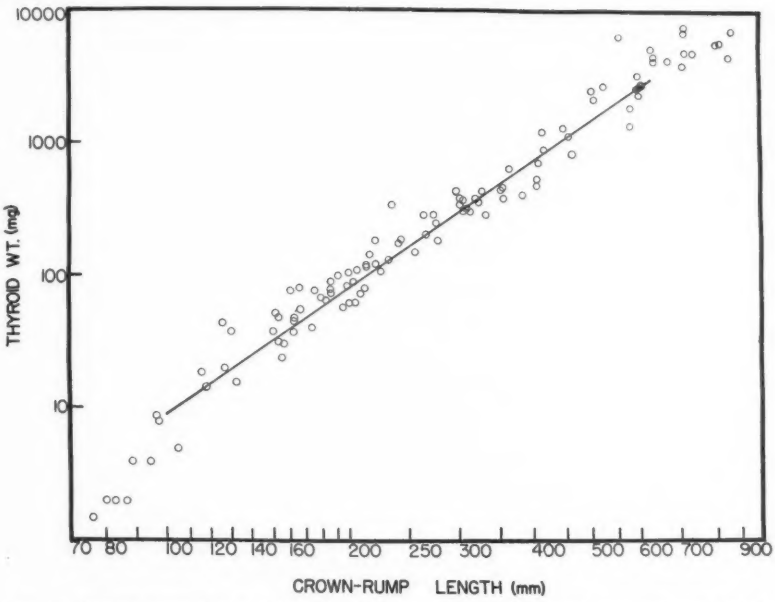

FIG. 2. Double-logarithmic plot of fetal thyroid weight against crown-rump length.

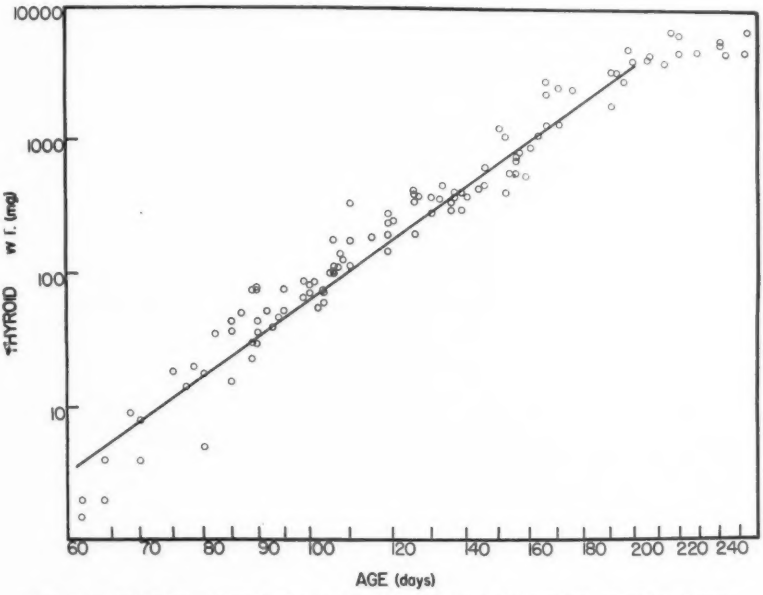

FIG. 3. Double-logarithmic plot of fetal thyroid weight against calculated age.

b (the initial growth index of Reeve and Huxley, 1945)
= a constant that indicates the value of y when x is 1, i.e., a scale factor.
k = the allometric growth constant.

Relation to Body Weight

In the case of fig. 1, equation (1) becomes

$$y = 0.25x^{1.0} \tag{2}$$

Equation (2) is valid from approximately 40 to 10,000 gm. body weight and from 11 to 3000 mg. thyroid weight.

The relative growth constant of the fetal thyroid with respect to body weight is 1.0. This indicates that during prenatal life, thyroid weight is almost directly proportional to body weight.

It is of interest to note that our value for k, namely 1.0, which is based on 121 measurements, is in good agreement with values reported earlier by Abeloos (1946) for calf fetuses (0.95), and by Schultze and Turner (1945) for fetal goat thyroids (0.98). Brody and Kibler (1941), furthermore, found a value of 0.924 for the relation of thyroid weight to body weight in a large variety of mature mammals.

Relation to Body Length

The expression for the curve shown in fig. 2 (thyroid weight plotted against C.R. length) is

$$y = (4.6 \cdot 10^{-6})x^{3.4} \tag{3}$$

The validity of this formula extends from 100 to 600 mm. C.R. length and from 10 to 4000 mg. thyroid weight.

Relation to Age

The curve representing thyroid growth in relation to age is shown in fig. 3. The following formula was found to fit the observed data:

$$y = (2.3 \cdot 10^{-11})x^{6.2} \tag{4}$$

This formula holds from 60 to 200 days of age with a corresponding thyroid weight range of from 2.7 to 4800 mg. Beyond 200 days of age there was considerable spread in the data.

The manner in which the values for the constants k and b were derived is shown below for fig. 3.

Let $x_1 = 60$ days and $x_2 = 200$ days; then y_1 (thyroid weight) = 2.7 mg. and $y_2 = 4800$ mg.
Therefore,

$$k = \frac{\log_{10} y_2 - \log_{10} y_1}{\log_{10} x_2 - \log_{10} x_1}$$

$$= \frac{\log_{10} 4800 - \log_{10} 2.7}{\log_{10} 200 - \log_{10} 60}$$

$$= 6.2.$$

The value for b was calculated from the expression $\log b = \log y_0 - k \log x_0$, where y_0 (organ weight) and x_0 (age in days) are values on the plot of thyroid weight against age from approximately 62 to 200 days. For example, at 200 days:

$$\log_{10} b = \log_{10} y_0 - k \log_{10} x_0$$

$$= \log_{10} 4800 - 6.2 \log_{10} 200$$

$$= 2.3 \times 10^{-11}$$

Percentage Growth Rate

The percentage growth rate of an organ can be obtained from the expression

$$\frac{d \text{ (organ weight)}/d \text{ (age)}}{\text{organ weight}}.$$

Employing the above notation, this expression becomes

$$\frac{dy/dx}{y}.$$

If the absolute rate of growth of the thyroid gland is dy/dx, then

$$\frac{d}{dx}(y) = \frac{d}{dx}(bx^k)$$

$$= bkx^{k-1}$$

$$= bx^k(kx^{-1}).$$

Therefore

$$\frac{dy}{dx} = y\frac{k}{x}$$

and

$$\frac{dy/dx}{y} = \frac{k}{x}$$

For example, in the calf at the age of 75 days, $[(dy/dx)/y] = (k/x) = (6.2/75) = 0.083$. Therefore, the rate of growth of the fetal calf thyroid at 75 days is 8.3 per cent per day, i.e., per cent of its weight at this age.

Fig. 4 shows that the percentage growth rate declines steadily with increasing age. Thus, at 60 days, the percentage growth rate (instantaneous relative growth rate $\times 100$) is 10.2 per cent per day; at 220 days, approximately 2.8 per cent per day. Such a decline has also been demonstrated by Lowrey (1911) for the fetal pig thyroid and by Fenger (1913) for the fetal calf thyroid. The curve in fig. 4

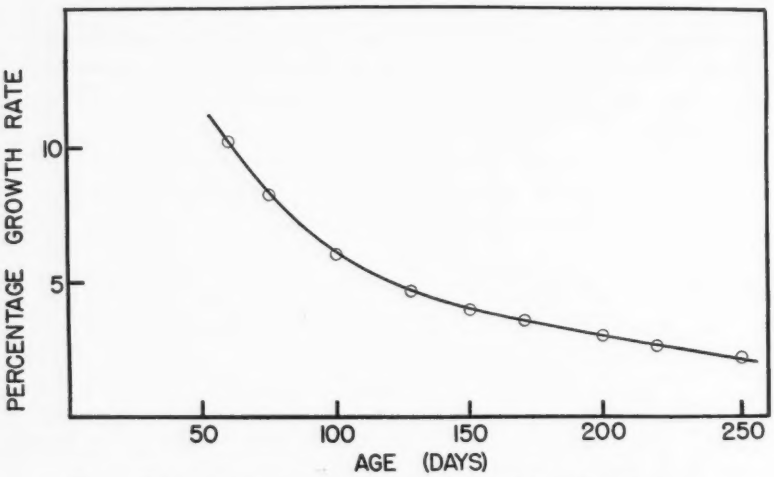

FIG. 4. Plot of percentage growth rate of the fetal thyroid against age.

resembles that obtained by Brody (1941) for the percentage growth of the thyroid in the postnatal state.

SUMMARY

The growth of the thyroid gland, in relation to body weight, body length, and age, in the bovine fetus from 62 days to term was investigated. Fetal age was calculated from the following four parameters: body weight, crown-rump length, chest circumference, and abdominal circumference.

The simple allometry equation $y = bx^k$ was found to fit the data for the growth of the thyroid in relation to body weight, body length, and age. The relative growth constant (k) for thyroid weight against body weight was found to be 1.0. This indicates that thyroid weight in the fetus is nearly directly proportional to body weight within the limits of the empirical formula.

Percentage growth rates were calculated and found to decrease with increasing age.

ACKNOWLEDGMENT

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THE ACCUMULATION OF THYROXINE-LIKE AND OTHER IODINE COMPOUNDS IN THE FETAL BOVINE THYROID[1]

J. WOLFF,[2] I. L. CHAIKOFF, AND C. W. NICHOLS, JR.
From the Division of Physiology of the University of California Medical School
BERKELEY

THE allometric constant (k in the equation $y = bx^k$) for thyroid weight as a function of body weight in the fetal calf was shown to be 1.0. This indicates that the growth of the thyroid gland in the fetal calf is almost directly proportional to body weight. Although the above formula was originally used in connection with morphological dimensions, it has been widely applied to chemical magnitudes by Needham (1942) who has termed such studies chemical heterogeny. Thus, in the allometric equation $y = bx^k$, y becomes the chemical entity and x the morphological magnitude (body weight, crown-rump (C.R.) length, or age in days).

In the present investigation, the accumulation of two chemical fractions, thyroxine-like and non-thyroxine iodine, was observed in the fetal calf thyroid from 53 days to term. The amounts found were correlated with body weight, C.R. length, and age of the fetus. The results obtained show that the accumulation of both iodine fractions in the fetal calf thyroid is related to the growth parameters (body weight and C.R. length) in a manner similar to that of the growth of the gland itself.

EXPERIMENTAL

Fetal calf thyroids were collected as described in the preceding paper. A total of 96 thyroids was obtained from fetuses ranging in age from 53 days to term. Fifty-five were males and 41, females. The dams were of the following breeds: Hereford, 82; Guernsey, 2; Holstein, 9; and Jersey, 3.

The thyroids were excised from the fetuses approximately one hour after death of the mothers. Representative portions of most of the glands were hydrolyzed on a steam bath for 12 hours in 2N NaOH (20 ml. per gm. wet weight). A suitable aliquot of the hydrolysate was analyzed for thyroxine-like and non-thyroxine iodine according to the method of Taurog and Chaikoff (1946).

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[2] U. S. Public Health Fellow.

In those cases in which organic and inorganic iodine were separated, the glands were homogenized in an all-glass apparatus with cold 10 per cent trichloroacetic acid (10 ml. per gram). The precipitate obtained after centrifugation was washed twice with cold five per cent trichloroacetic acid. The supernatants, which contained all of the inorganic fraction, were combined and analyzed as previously described (Taurog and Chaikoff, 1946b). The trichloroacetic acid soluble (organic) fraction was hydrolyzed in 2N NaOH, and treated as described in the preceding paragraph.

RESULTS

Thyroid iodine of 16 fetuses was separated into trichloroacetic acid-soluble and trichloroacetic acid-precipitable iodine. The results are recorded in table 1. The trichloroacetic acid-soluble fraction constituted, as a rule, 11 per cent or less of the total iodine. In only one case was a value above 11 per cent found. It is evident, therefore,

TABLE 1. THE ORGANIC AND INORGANIC FRACTIONS OF FETAL CALF THYROIDS

Calculated fetal age	Thyroid weight	Iodine		
		Organic	Inorganic	Inorganic as per cent of total iodine
days	mg.	gamma	gamma	
64	1	0.94	0.10	9.6
68	9	1.2	0.22	18.5
75	9	0.84	0.10	10.6
78	17	1.0	0.08	7.6
80	18	3.4	0.34	9.1
85	24	0.98	0.08	7.5
107	133	14.6	0.24	1.6
108	111	17.0	0.21	1.2
120	255	25.4	2.6	9.3
125	345	55.2	2.2	5.9
143	895	129	12.1	8.5
154	$1.13 \cdot 10^3$	120	14.6	10.8
168	$2.37 \cdot 10^3$	424	33.9	7.4
172	$2.63 \cdot 10^3$	360	34.2	8.6
198	$4.32 \cdot 10^3$	$2.54 \cdot 10^3$	198	7.2
252	$8.00 \cdot 10^3$	$2.59 \cdot 10^3$	76.5	2.8

TABLE 2. NATURE OF THYROID IODINE OF THE ADULT BOVINE
(All females approximately 6 years old)

Thyroid weight	Iodine Contents		
	Organic	Inorganic	Inorganic as per cent of total iodine
gm.	mg.	gamma	
16.2	25.9	450	1.7
17.0	14.9	259	1.7
14.0	15.3	305	1.5
18.0	34.1	345	1.0

TABLE 3. NATURE OF IODINE IN FETAL CALF THYROID

Age range	Number of fetuses examined	Fetal weight		Thyroid							
				Weight		Total iodine		Thyroxine iodine		Non-thyroxine iodine	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
days		gm.	gm.	mg.	mg.	gamma	gamma	gamma	gamma	gamma	gamma
53– 59	3	6.7 –12.7	8.3	—	—	<0.1 – 0.40	—	<0.1	—	<0.1 – 0.35	—
60– 68	6	12.7 –34	24	1– 9	3.4	0.91– 1.2	0.95	0.1 – 0.36	0.15	0.58– 1.0	0.70
70– 80	6	34– 67	52	4– 19	12.5	0.26– 3.8	1.9	0.1 – 0.79	0.44	0.51– 2.9	1.34
82– 88	8	79–156	119	16– 78	38	1.8 – 7.8	4.1	0.42 – 3.5	1.53	0.56– 5.8	2.6
90– 98	7	96–240	179	37– 78	54	3.3 –15.5	6.0	0.5 – 4.0	1.4	2.8 –11.5	4.7
100–105	9	285–490	371	57–183	106	4.6 –20.0	9.9	0.95 – 5.0	2.7	3.6 –15.0	8.3
106–110	7	438–555	491	110–178	131	7.7 –26.6	16.4	1.7 –11.1	4.6	4.6 –18.8	11.8
		kg.	kg.								
118–125	5	0.71– 1.32	0.89	155–435	243	42.3 –63.9	49.7	9.8 –38.3	17.9	25.1 –39.6	31.8
126–138	9	0.14– 1.75	1.44	290–470	379	26.6 –127	96.6	15.8 –36.6	26.5	10.8 –91.2	70.0
				gm.	gm.						
140–160	12	1.80– 3.81	2.54	0.42– 1.13	0.75	135–423	252	28.0 –118	60.6	82.5 –351	189
163–170	6	4.34– 5.50	4.81	1.13– 2.52	1.87	315–805	580	75.4 –300	212	190–550	362
						mg.	mg.	mg.	mg.	mg.	mg.
172–205	6	5.63– 9.60	8.55	2.43– 5.07	3.56	0.40– 2.74	1.95	0.078– 1.01	0.51	0.29– 1.88	1.40
215–218	3	12.5 –15.7	14.3	4.71– 6.95	6.02	3.21– 5.56	4.27	1.07 – 1.54	1.35	2.14– 4.02	3.00
240–255	6	19.9 –26.8	23.7	4.60– 8.00	5.97	2.67– 9.20	6.28	0.69 – 2.63	1.88	1.90– 6.58	4.38
260–265	2	32.5 –40.4	—	10.3 –11.4	—	4.96–17.4	—	1.40 – 5.32	—	3.56–12.1	—

that the major proportion of thyroid iodine in the fetus is organic. The values obtained by trichloroacetic acid separation of the adult bovine thyroid are shown in table 2.

The iodine values for thyroxine and non-thyroxine in the fetal thyroids have been arbitrarily grouped in table 3 according to the calculated ages of the fetuses. The values for the new-born calf are recorded in table 4.

TABLE 4. NATURE OF THYROID IODINE OF NEW-BORN CALVES
(All males)

Thyroid weight	Iodine contents		
	Total iodine	Thyroxine-like iodine	Non-thyroxine iodine
gm.	mg.	mg.	mg.
6.95	6.76	2.08	4.68
7.21	8.48	2.40	6.08
2.63	3.16	0.93	2.23
4.51	6.42	1.60	4.82
4.00	4.42	1.04	3.38

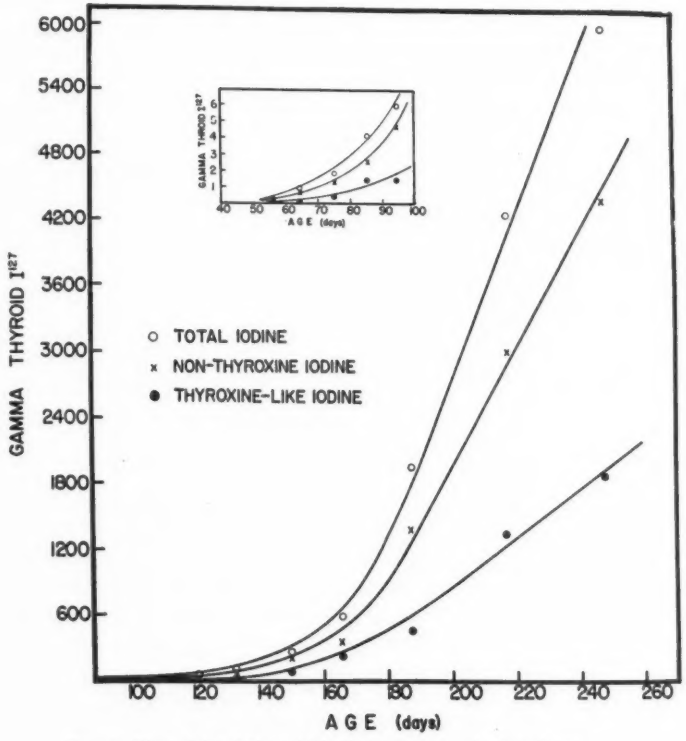

FIG. 1. Plot of fetal thyroid iodine against calculated age.

Despite considerable variation in the individual values for each age group, the means show that the iodine content of the fetal thyroid increases steadily with increasing body weight, C.R. length, and calculated age (table 3).

The average values for thyroxine-like and non-thyroxine iodine are plotted against calculated age in fig. 1. The curves suggest an ex-

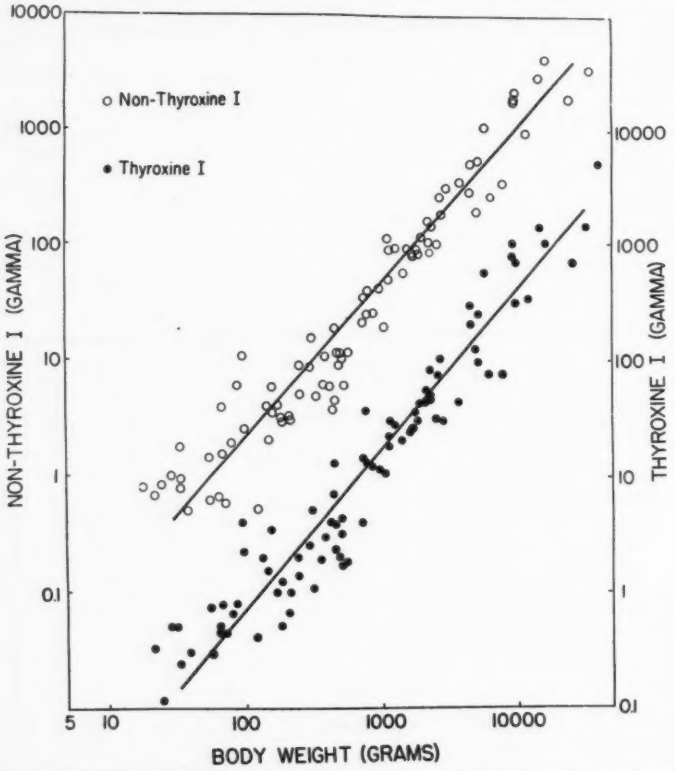

FIG. 2. Double-logarithmic plot of fetal thyroid iodine against body weight.

ponential relation between these iodine fractions and the age parameter. The chemical entities (thyroxine-like, non-thyroxine iodine) were therefore plotted against body weight, C.R. length, and age (figs. 2–4) on a double logarithmic grid. The straight-line curves obtained were subjected to analysis by application of the allometry equation of Huxley (1932), $y = bx^k$.

Relation to Body Weight

The equation for thyroxine-like iodine as a function of body weight (fig. 2) is

$$y_1 = (1.2 \cdot 10^{-3})x^{1.4} \quad (1)$$

where y_1 = thyroxine-like iodine in gamma.
x = body weight in gm.
The constants b ($1.2 \cdot 10^{-3}$) and k (1.4) were derived as described in the preceding paper.

Likewise, the equation for non-thyroxine iodine against body weight shown in fig. 2 is

$$y_2 = (3.1 \cdot 10^{-3})x^{1.3} \quad (2)$$

where y_2 = non-thyroxine iodine in gamma,
x = body weight in gm.

These formulae are valid for a body weight range from 50 gm. to 20 kg.

The constants expressing the "growth" of the chemical entities (1.4 for thyroxine-like iodine; 1.3 for non-thyroxine iodine) indicate that thyroxine-like and non-thyroxine iodine increase at approximately the same rate in the growing calf fetus.

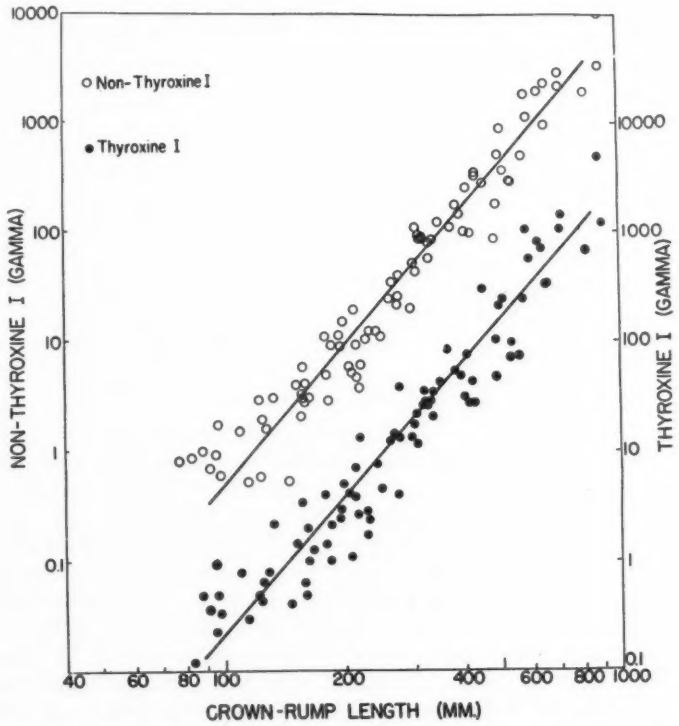

FIG. 3. Double-logarithmic plot of fetal thyroid iodine against crown-rump length.

Grown-Rump Length Relations

The curves for thyroxine-like and non-thyroxine iodine plotted against C.R. length are shown in fig. 3. The following equation fits the data for thyroxine-like iodine against C.R. length:

$$y_1 = (1.3 \cdot 10^{-9})x^{4.1} \quad (3)$$

in which x = C.R. length in mm.

y_1 = thyroxine-like iodine in gamma.

The formula for non-thyroxine iodine plotted against C.R. length is

$$y_2 = (2.1 \cdot 10^{-9})x^{4.2} \quad (4)$$

The above expressions are valid for C.R. lengths extending from 100 to 800 mm.

A comparison of the two equations (where k is 4.1 and 4.2, respectively) indicates that thyroxine-like and non-thyroxine iodine increase at about the same proportion in relation to body length.

Age Relations

A plot of thyroxine-like and non-thyroxine iodine against age yields curves having the following formulae

$$y_1 = (5.7 \cdot 10^{-10})x^{5.4} \quad (5)$$

$$y_2 = (1.8 \cdot 10^{-10})x^{5.3} \quad (6)$$

where y_1 and y_2 represent thyroxine-like and non-thyroxine iodine, respectively, in gamma, and x, the age in days. The validity of the formulae for thyroxine-like and non-thyroxine iodine against age extend from 60 days to term.

The amounts of these two iodine fractions in the fetal calf thyroid increase at approximately the same proportion with increasing fetal age. The values for k_1 and k_2 were 5.4 and 5.3, respectively.

DISCUSSION

As the fetus grows, total and thyroxine-like iodine increase steadily in the gland, and the rate at which each of these iodine fractions accumulates is shown to bear an exponential relation to body weight and body length as well as to calculated age. The accumulation of these two iodine fractions in the fetal gland is not only the result of thyroid growth (which, as shown in the previous paper, is also exponential) but, in addition, appears to be the result of an actual increase in the capacity of thyroid tissue to store iodine with increasing age. This increasing iodine-storage capacity of thyroid tissue, with age, is shown in fig. 5 which shows the *concentration* of iodine fractions in the gland (mg. iodine per 100 gm. of fresh tissue) against fetal age.

It was previously demonstrated, in 11 vertebrates, including the rat, examined under a variety of experimental conditions, that thyroxine iodine, when expressed as a percentage of total iodine of the gland, remains fairly constant (Wolff *et al.*, 1947; Taurog *et al.*, 1946c, 1946d). The values for this percentage ranged from 25 to 32. It therefore became of interest to determine whether a similar relation existed in the fetal calf thyroid.

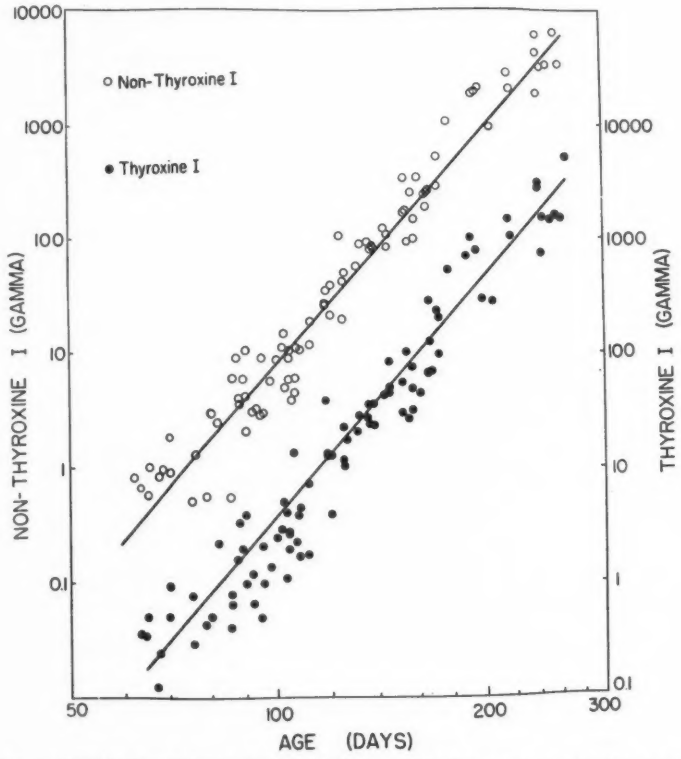

FIG. 4. Double-logarithmic plot of fetal thyroid iodine against calculated age.

The parallelism between the two curves shown in each figure (2, 3, 4, and 5) suggests that in the fetal calf, also, thyroxine iodine comprises a constant proportion of total thyroid iodine. An examination of the ratios for individual thyroids of fetuses obtained during the last third of the period of gestation revealed a constancy in the proportion of thyroxine-like iodine to total iodine. Eighteen specimens that were 170 days or older were studied, and with the exception of a single ratio, all values fell within the range of 24 to 36 per cent. This constancy in the proportion of thyroxine iodine to total iodine was

first observed with frequency, in fetuses older than 120 days. But in younger fetuses, the proportion varied considerably, most of the values falling below 20 per cent. The deviations from 24–36 per cent were equally divided between the sexes and did not appear to be related to the absolute amounts of iodine contained in these glands.

Rankin (1941) investigated the appearance of iodine in the thyroid of the fetal pig, which has a gestation period of 114 days. He first found inorganic iodine in the gland between 46 and 50 days of age, and organic iodine at 52 days of age. As shown here, thyroxine-

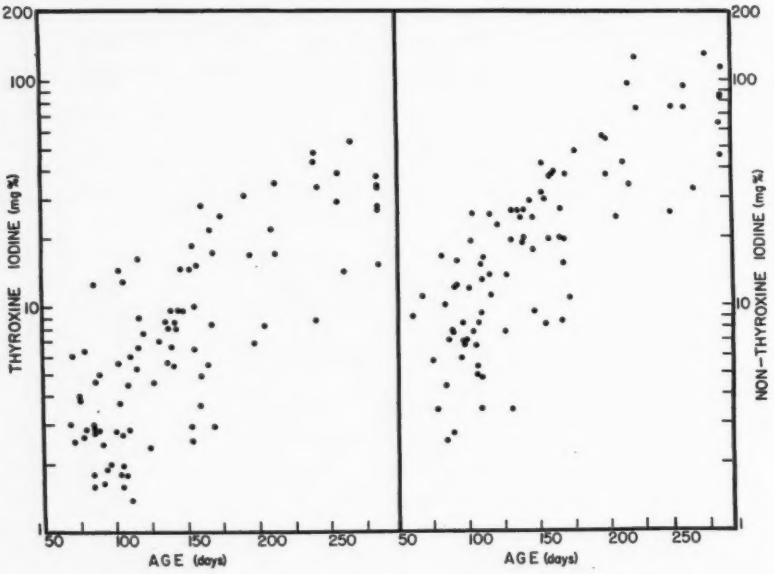

FIG. 5. Semi-logarithmic plot of the concentration of fetal thyroid iodine against calculated age.

like iodine appears at an earlier stage of gestation in the fetal calf, being first detected at 60 days of age (gestation period, 278–285 days).

Although the present investigation clearly demonstrates that thyroxine-like and non-thyroxine iodine increase proportionately with increasing fetal size and age, it provides no information on the nature of the iodine taken up by the fetal thyroid gland. It is unlikely that the iodine was taken up in the form of thyroxine or diiodotyrosine (Leblond, 1942). The iodine probably enters the fetal gland in the inorganic form as it does in the adult. Gorbman and Evans (1943) have demonstrated, in the fetal rat, that functional activity of the thyroid gland (as judged by its capacity to concentrate radioactive inorganic iodine) begins at 18–19 days of gestation. Preliminary experiments with surviving slices of fetal calf thyroid have shown that

this tissue also has the capacity to concentrate inorganic iodine and that this capacity appears at a considerably earlier stage in the developing fetal calf than in the rat fetus.

SUMMARY

The amount of inorganic, thyroxine-like, and non-thyroxine iodine were measured in the bovine fetal thyroid from 53 days to term.

Measurable amounts of iodine were first detected in the fetal thyroid at 60 days of age.

The percentages of total iodine present as inorganic were similar to those observed in the adult thyroid gland.

The accumulation of thyroxine-like and non-thyroxine iodine in the fetal thyroid is related to each of the following growth parameters: body weight, crown-rump length, and calculated age. A straight-line relationship was found to exist when these iodine fractions were plotted against each of the parameters on a double-logarithmic grid.

The amounts of iodine found in the bovine fetal thyroid with increasing age were greater than could be accounted for by mere increase in thyroid mass. A progressive increase in the iodine concentrating capacity of fetal thyroid tissue occurred with age.

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RELATIONSHIPS OF DIET TO THE DURATION OF SURVIVAL, BODY WEIGHT AND COMPOSITION OF HYPOPHYSECTOMIZED RATS[1]

JAMES H. SHAW AND ROY ORVAL GREEP
From the Harvard School of Dental Medicine, and the Department of Nutrition, Harvard School of Public Health
BOSTON, MASS.

SURGICAL removal of the pituitary gland has been known for many years to be compatible with life but investigators using such pituitary-less mammals have learned with dismay that this is a qualified truth. Animals lacking this gland are at best frail creatures with no ability to adapt themselves either to physiological stresses nor to environmental vicissitudes. Their survival is possible only under controlled laboratory conditions.

In this laboratory, large numbers of hypophysectomized rats have been used for a variety of experimental procedures in which no particular attention has been paid to their diet other than to offer them laboratory chow *ad libitum*. Under these circumstances, it has been repeatedly observed that a variable mortality had to be reckoned with in both treated and control groups in experiments lasting much beyond 10 days after operation. For example, in one untreated group of 13 hypophysectomized young male rats, 10 survived until the 8th post-operative day, and 6 until the 22nd day; in another untreated group of 18 young male hypophysectomized rats, only 8 survived until the 30th day. In a third untreated group of 12 male rats, 7 died before the 15th post-operative day and an additional 4 within the next 28 days (Greep and Deane, unpublished data). In striking contrast, preliminary observations in this laboratory indicated that when hypophysectomized rats were maintained on purified rations adequate in all known nutritional essentials, a high percentage survived for several weeks, and the general appearance of the animals remained quite good. Since many experiments in which hypophysectomized rats are used would benefit by long periods of survival, any experimental regimen on which a high percentage of the operated rats could be maintained consistently for several weeks or months would be of distinct value.

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This preliminary investigation was designed to test the extent of the differences in the percentage survival and in the duration of survival when hypophysectomized male rats were maintained on commercial laboratory chow and on purified diets. In addition, tests were made to determine whether some of the more obvious physical and chemical differences between laboratory chow pellets and granular purified rations were responsible for their different effects on the well being of the hypophysectomized rat.

EXPERIMENTAL PROCEDURE

In the first experiment, 46 male albino rats of the Sprague-Dawley strain, which were 28 days of age, were hypophysectomized by the parapharyngeal approach during one day. Then, the animals were immediately distributed into 5 groups, in such a way that the range and average body weights of the rats in any group were almost identical to the respective values in any other group. Each rat was placed in a small individual cage with wire bottom and supplied with water *ad libitum* by a freely flowing watering bottle. One group was fed a commercial laboratory chow ground sufficiently fine to pass readily through a 20-mesh sieve, thereby approximating the particle size of the purified rations. This was deemed necessary as a test of whether the hard pellets of laboratory chow were sufficiently difficult for lethargic, hypophysectomized rats to nibble and masticate as to reduce their food consumption to a sub-optimal amount. Another group was offered the same chow in its original pellet form.

The other 3 groups were given purified rations, the basic components of which are recorded in Table 1. These diets are adequate for the normal rat in all known nutritional essentials. One of these groups received a high carbohydrate ration (100) which had an energy value of about 4.1 calories

TABLE 1. COMPOSITION OF THE PURIFIED RATIONS FED TO HYPOPHYSECTOMIZED RATS

Ration components	Ration		
	100	100+20% cellu flour	150
	gm.	gm.	gm.
Sucrose	67	67	31
Casein[1]	24	24	24
Salts[2]	4	4	4
Corn oil[3]	5	5	5
Lard	—	—	16
Cellu flour[4]	—	20	—
Whole liver substance[5]	2	2	2
1:20 liver concentrate powder[5]	2	2	2

[1] Casein—"De-vitaminized" casein (Sheffield Farms, New York, N. Y.) to each 24 gm. of which has been added 350 μg. thiamine hydrochloride, 350 μg. riboflavin, 350 μg. pyrixodine hydrochloride, 2.5 mg. nicotinic acid, 2.0 mg. calcium pantothenate, 100 mg. choline chloride, 100 mg. inositol and 30 mg. para-aminobenzoic acid.

[2] Corn oil—Mazola to 5 gm. of which has been added 1.1 mg. beta-carotene, 300 I.U. irradiated ergosterol, 0.6 mg. 2-methyl-1, 4-naphthoquinone and 5.0 mg. alpha-tocopherol.

[3] J. Dental Research 26, 47 (1947).

[4] Chicago Dietetic Supply House, Chicago, Ill.

[5] The Wilson Laboratories, Chicago, Ill.

per gram. A second group was given the same ration to which had been added 20 per cent Cellu flour as a source of crude, undigestible fiber (100+ 20 per cent Cellu flour); the energy value of this ration was about 3.5 calories per gram, which closely approximated the energy value of the laboratory chow. It has been found that the addition of this amount of crude fiber to purified rations does not appreciably affect its nutritional properties when the diet is offered *ad libitum*.

As a reverse evaluation of any possible energy relationship, the energy value of the purified ration per gram was increased by the isocaloric replacement of lard for part of the sucrose. Thus a third group was offered ration 150 in which approximately one-half of the carbohydrate had been replaced by lard (16 grams of lard substituted for 36 of each 67 grams of sucrose). This ration had a caloric value of about 5.1 calories per gram. All diets were made available to the rats *ad libitum*.

At the end of 88 days, the 16 surviving animals were killed with chloroform and the weights of the adrenal, thymus, testes, seminal vesicles and testicular fat bodies determined on a Roller-Smith torsion balance. Ratios of weights of these organs to body weight were calculated as indices of the activity of the related endocrine systems. The sella turcica of each animal was examined for evidence of any remnants of the hypophysis. Radiographs were made of the tails of 1) the survivors in experiment 1; 2) 10 rats with body weights comparable to those of the survivors at the time of hypophysectomy; and 3) 10 rats with body weights corresponding to the autopsy weights of the hypophysectomized rats. From the latter group, the organs mentioned above were weighed.

The average length of the last 22 caudal vertebrae, the 12th caudal vertebra and the 12th caudal intervertebral space were measured for the survivors and the controls as tests of whether tail growth had occurred.

In the second experiment, 34 male albino rats of the same strain and age were hypophysectomized on the same day and immediately divided into three similar groups which were housed as in the first experiment and fed ground chow, the high carbohydrate purified diet (100) and the high fat purified diet (150) *ad libitum*, respectively. In this case, the food consumption per rat per day was determined for the first 70 days of the experiment. On the 112th day, 4[2] of the 14 survivors were saved for longevity studies and for histologic purposes; the remaining 10 were sacrificed and the same organs weighed as in the first experiment. All these tissues except those needed for histological study were placed with the rest of the carcass after weighing; then analyses of the carcasses minus the contents of the gastrointestinal tract, were made for water, fat, ash and protein by the methods described by Deuel, Hallman, Movitt, Mattson and Wu (1944). The tails of these rats were radiographed and the same measurements made as in the initial experiment.

The testes and adrenals of representative rats from the two surviving groups of the second experiment were fixed in 10 per cent neutral formalin. Paraffin sections of the testes were stained in hematoxylin and eosin. Frozen sections of one adrenal from each animal were stained with 1) Sudan IV, 2)

[2] The four rats which were not sacrificed at 112 days were continued on their original experimental regimens. One died on the 176th post-operative day, a second was sacrificed in a weakened state on the 240th day and 2 are still alive on the 260th day.

Sudan IV and Harris' hematoxylin and 3) by the Schiff plasmal method. An additional two sections of each adrenal were mounted unstained in glycerin jelly, one was untreated, the other extracted for one-half hour in acetone at room temperature. These latter were studied under the polarizing microscope for acetone-soluble droplets showing birefringence and under the flourescent microscope for the presence of autofluorescing material.

All animals in both experiments were weighed every other day throughout the experimental periods. Each morning, the numbers of animals which had died in the previous 24 hours were recorded. At frequent intervals, all animals were palpated for gross evidence of increase in testicular size. In all, 4 rats were discarded from further consideration when slight testicular enlargement was noted. Any animals which died in the first 48 hours after the operation were considered to have died because of trauma incurred in the operation and not because of hypophysectomy *per se*. Of the 80 rats in these experiments, 5 died within the first 48 hours and were disregarded in all evaluations of the data.

RESULTS

The general appearance of the hypophysectomized rats maintained on the purified rations was exceptionally good throughout the duration of the experimental periods. The hair coat remained fine and smooth in texture. The external appearance of the hypophysectomized rats maintained on the high fat ration (150) was especially sleek and well-groomed. No evidence of cyanosis at the extremities was observed except during short periods previous to death in those which

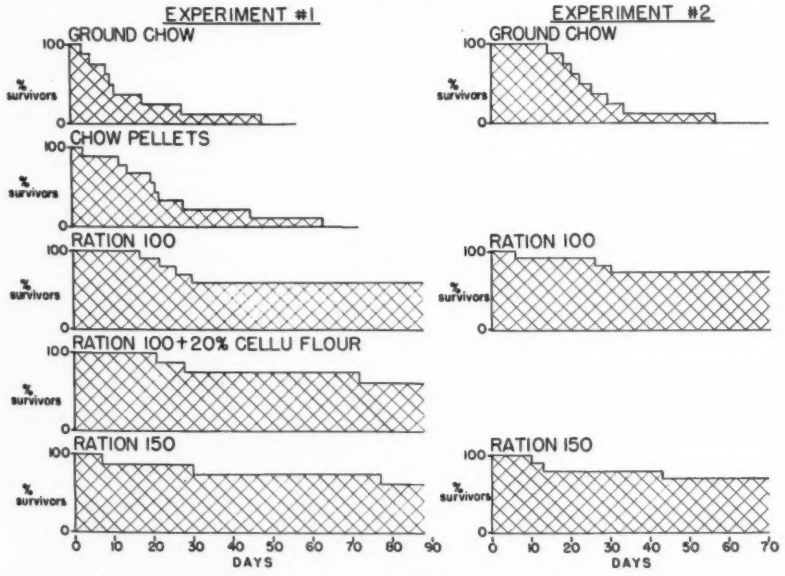

FIG. 1. Graphical representation of the percentage survival of hypophysectomized rats maintained on the various rations in experiments 1 and 2.

TABLE 2. NUMBER OF SURVIVORS AND THEIR CHANGES IN BODY WEIGHT AS OF THE 7TH, 35TH, AND 70TH POST-OPERATIVE DAYS AND AT THE END OF THE EXPERIMENTS

Ration	Initial no. of rats*	Avg. wt.	7th post-operative day			35th post-operative day			70th post-operative day			88th post-operative day		
			No. survivors	Avg. wt. increase	Range of wt. change	No. survivors	Avg. wt. increase	Range of wt. change	No. survivors	Avg. wt. increase	Range of wt. change	No. survivors	Avg. wt. increase	Range of wt. change
		gm.		gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.
Experiment 1														
Ground chow	8	57	6	6	−6 to 16	1	15	—	0	—	—	0	—	—
Chow pellets	9	59	8	15	9 to 19	2	15	14 to 15	0	—	—	0	—	—
100	10	64	10	6	−7 to 16	6	13	−2 to 29	6	38	7 to 55	6	43	7 to 63
100+20% Cellu flour	8	64	8	8	−7 to 14	6	20	−9 to 32	6	23	−1 to 33	5	28	4 to 41
150	8	61	7	12	3 to 20	6	24	14 to 37	6	37	20 to 58	5	49	38 to 69
Experiment 2												112th post-operative day		
Ground chow	8	50	8	9	−1 to 28	1	13	—	0	—	—	0	—	—
100	11	50	10	11	−3 to 19	8	27	7 to 50	8	39	13 to 76	7	55	40 to 93
150	10	48	10	9	−7 to 25	8	24	6 to 41	7	39	7 to 61	7	52	24 to 76

* Rats which were found to have partial testicular maintenance during the experimental period, a remnant of the hypophysis at post-mortem, or which died from operative trauma were not included in these numbers.

did not survive the experimental periods. In contrast, within a few hours after hypophysectomy, the rats fed either ground chow or chow in pellet form began to appear unkempt and cyanotic and often remained thus until death. The animals fed chow as pellets or in ground form were also extremely lethargic in contrast to the relative activity of those rats maintained on the purified ration. No difference was noted in behavior or general appearance between the rats on the two forms of chow.

The effect of the various diets upon the duration of survival of hypophysectomized rats is presented in Figure 1 and in Table 2. In experiment 1, the duration of survival and the percentage of survivors among the 3 groups of rats fed the purified rations (100, 100+20 per cent Cellu flour, and 150) were significantly higher than among the groups of rats fed chow pellets or ground chow. No significant differences were observed in the survival times and percentage of survivors among the 3 groups of rats fed the different purified rations, nor were any significant differences observed between the groups fed the two physically different forms of chow. In experiment 2, a significantly longer duration of survival and a significantly higher percentage of survivors was observed among the hypophysectomized rats fed the 2 purified rations than among those rats fed ground chow. Again, no differences were observed in survival between the groups fed the two purified rations.

During the first few days after hypophysectomy, numerous rats in each of the dietary groups lost appreciable amounts of body weight while others in each group maintained their original weight and some began to gain immediately. On the 7th day of the first experiment, the average weight increase for each group varied from 6 to 15 grams. At this time, the rats fed chow in pellet form and those fed ration 150 had increased in body weight on the average slightly more than those animals in the other 3 groups. On the 35th day, the 6 surviving animals fed ration 150 had gained on the average slightly more than the 6 fed 100+20 per cent Cellu flour and these in turn had gained slightly more than the surviving 2 rats in the ground chow group, the 1 survivor in the chow pellet group and the 6 rats in the ration 100 group. By the 70th day and 88th days, there were no survivors in the groups receiving chow but those hypophysectomized rats fed rations 100 and 150 were equal in average weight gain and had gained appreciably more than those rats fed the ration with added undigestible crude fiber. The average increase in body weight for the rats fed rations 100 and 150 for 88 days was slightly in excess of two-thirds of the body weights at the time of hypophysectomy.

In experiment 2, the average weights of the rats in the 3 groups on the 7th day post-operatively were 9 to 11 grams more than at the time of the operation. By the 35th day, the average weight increases in rats fed the purified rations were double that observed in the one

survivor in the group fed ground chow. On the 70th and 112th post-operative days, the average weight increase of the surviving hypophysectomized rats fed ration 100 was almost identical with that of those rats fed ration 150 throughout the experimental period. It is interesting to note that the average weight of the rats fed rations 100 and 150 for 112 days was slightly more than double the average initial body weights.

Food consumption expressed as the average number of calories consumed per rat per day was almost identical for the 2 groups fed the purified rations and slightly exceeded that of the rats fed ground chow.

The average ratios of organs to body weight, expressed as mg. per 100 gm. of body weight, are presented in table 3 for the survivors of experiment 1 and for the 10 animals in experiment 2 which were

TABLE 3. THE AVERAGE PROPORTION OF TISSUE WEIGHTS TO BODY WEIGHTS EXPRESSED AS MG. PER 100 GM. OF BODY WEIGHT

Exp. no.	Duration days	Ration	No. of rats	Body weight		Adrenal prop.	Thymus prop.	Testes prop.	Seminal vesicle prop.	Testicular fat prop.
				Initial	Final					
				gm.	gm.					
1	88	100	6	63	104	8.4	229	120	7.0	680
		100+20% Cellu flour	5	63	89	8.2	204	137	8.5	590
		150	5	63	108	8.8	234	121	7.9	1070
2	112	100	5	50	107	10.5	190	67	6.0	815
		150	5	52	104	10.1	160	100	6.9	871
Control	0	Chow	10	—	107	21.8	455	962	34.9	355

sacrificed for carcass analysis on the 112th day. These data do not include any animals on chow for the reason that none survived the experimental period. The very low ratios of the testes and of the seminal vesicles to body weight reflect the withdrawal of gonadotropic hormones due to the complete removal of the hypophysis and indicate further that this atrophic process is not influenced by the nutritional state of the animal which has permitted his survival for this length of time. The average percentile weight of the adrenals is also far below the range for intact animals of this weight but is not quite as low as that observed previously by Deane and Greep (1946) in hypophysectomized rats surviving for 56 and 136 days on chow. The average ratio of the weight of the thymus to body weight had not been reduced below the normal range for animals of equal weight.

In table 4 are recorded measurements taken from radiograms of the tails of 2 different groups of 10 intact control animals and of animals surviving on the purified diets for 88 and 112 days. The average length of the last 22 tail vertebrae in the hypophysectomized rats increased appreciably during the experimental period (Fig. 5 & 6). This increase in length is almost the same as that which occurred in normal rats during the same weight increase achieved during a shorter growth period and is apparently a result of the increase

TABLE 4. MEASUREMENTS ON THE AVERAGE LENGTH OF THE TAILS OF HYPOPHYSECTOMIZED RATS AND THEIR RESPECTIVE CONTROLS

Experiment	Ration	No. of rats	Body weight		Avg. length of the last 22 tail vertebrae	Length	
			Initial	Final		12th caudal vertebra	12th caudal intervertebral space
			gm.	gm.	mm.	mm.	mm.
1	100	6	63	104	106	5.5	.5
	100+20% Cellu flour	5	63	89	99	5.2	.4
	150	5	63	108	104	5.3	.5
2	100	5	50	107	113	5.9	.4
	150	5	52	104	106	5.7	.3
Control	Chow	10	—	64	78	3.5	.8
Control	Chow	10	—	107	106	5.3	.6

in length of the individual ossified vertebra. The distance between the adjacent vertebrae appears to be slightly longer in normal than in hypophysectomized rats of the same weight.

In table 5 are presented the carcass analyses for water, protein, fat, ash and carbohydrate for 2 groups of 5 rats, each of which were maintained for 112 days on ration 100 and 150, respectively. In addition, corresponding analyses are presented for these components in the carcasses of normal rats which had been maintained for 126 days on purified diets of comparable fat content by Scheer, Straub, Fields, Meserve, Hendrick and Deuel (1947). Values for normal rats fed a stock ration *ad libitum* are also listed from data of the latter investigators. The average analyses of the carcasses of the 2 groups of hypophysectomized rats do not differ appreciably from each other

TABLE 5. THE BODY COMPOSITION OF RATS FED PURIFIED RATIONS

Ration no.	Duration	Fat content of diet	No. rats	Average body weight	Water	Protein*	Fat	Ash	Carbohydrate
	days	%		gm.	%	%	%	%	%
				Hypophysectomized Rats					
100	112	5	5	105	57.5	15.2	19.4	3.9	4.0
150	112	25	5	103	57.7	14.3	20.4	3.9	3.7
				*Intact Rats***					
61	126	5	7	311	57.0	18.7	19.4	3.3	1.6
63	126	20	8	377	52.2	17.9	24.7	3.2	2.0
Stock	126	14	7	310	58.8	18.2	9.9	3.1	10.0

* We wish to acknowledge our gratitude to the Department of Nutrition of the Harvard School of Public Health for making the protein analyses of the dried, defatted carcasses of the hypophysectomized rats.

** These analyses quoted from Scheer *et al.*, J. Nutrition 34, 581 (1947).

except for the fat and protein components. The fat content of the rats fed high fat ration 150 was 20.4 per cent, whereas the fat content of the rats fed low fat ration 100 was 19.4 per cent. The average protein content of the rats fed rations 100 and 150 were 15.2 and 14.3 per cent, respectively. The carcass analyses of the hypophysectomized rats were very similar to comparable analyses of intact rats fed a low fat purified ration, except that the protein content for the 2 groups of hypophysectomized rats was appreciably lower than the 18.7 per cent value for intact rats. The carbohydrate content of the hypophysectomized rats was slightly higher than for the intact rats. Intact rats which were fed the high fat diet had a considerably higher fat content and lower water content than either group of hypophysectomized rats or than the intact rats fed the low fat purified diet. On the other hand, intact rats which were fed a stock diet composed of natural foodstuffs with an overall fat content of 14 per cent had an average carcass fat analysis of only 9.9 per cent. This low fat content of normal rats fed a stock ration indicates that the small amount of depot fat observed in hypophysectomized rats maintained in this laboratory on chow is probably a characteristic of the ration and not a result of hypophysectomy. Likewise the high fat content of the hypophysectomized rats and of the intact rats fed purified rations was apparently a result of the composition of the ration and not of the particular endocrine status of the rats *per se*.

HISTOLOGY

The hypophysectomized rats which were given the purified rations over extended post-operative periods had the appearance of exceptionally fat animals (Fig. 2); the heavy deposits of subcutaneous fat made them appear to be padded. The skin was pinkish and glistening and could be incised with the ease of parchment. When these animals were brought to necropsy, it was revealed by gross inspection that their carcasses were indeed laden with massive deposits of both white and brown fat (Fig. 3). The testicular fat bodies were enormous. They extended the full length of the abdominal cavity and displayed many long finger-like projections which ramified amongst the loops of the intestines. Masses of brown fat were found in the interscapular region and along the posterior wall of the thoracic and abdominal cavities. Here it was in such abundance as to obscure the great vessels (Fig. 4) and the adrenal glands. The amount of brown fat in the hypophysectomized rats fed purified rations was appreciably more than in hypophysectomized rats fed chow but was approximately equal to the amount observed to be present in intact rats fed these purified rations for similar periods.

The adrenal glands from these same animals show a severe reduction of the width of the cortex (Fig. 7 & 8). The zona fasciculata was so atrophic as to make its recognition doubtful. The cells in the

FIG. 2. A demonstration of the increase of body size of a typical hypophysectomized rat from experiment 2. The rat on the left has been maintained on ration 150 for 140 days after hypophysectomy and weighs 133 grams. The rat on the right is a normal one from the stock colony and weighs 78 grams which is the weight at which the rat on the left was hypophysectomized.

FIG. 3. Photograph of the abdominal cavity of a typical rat in experiment 2 which had been fed ration 150 for 112 days after hypophysectomy. Note the atrophic testes and the plentiful supplies of depot fat.

FIG. 4. Photograph of the thoracic cavity of the rat in figure 3 with lungs removed to show the extensive brown fat body along the dorsal aorta.

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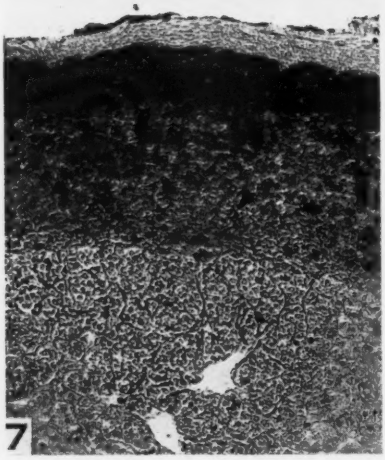
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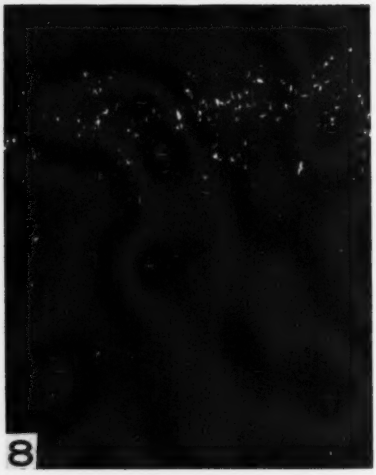
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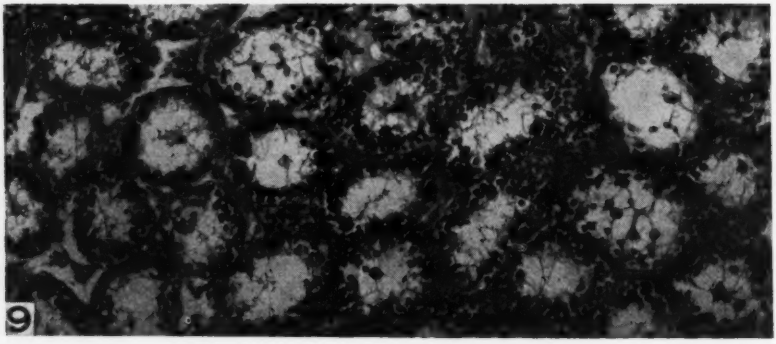
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area between the zona glomerulosa and the medulla were shrunken, had pycnotic nuclei and were devoid of sudanophilic droplets. This area did not respond to the Schiff plasmal reagent nor did it contain any autofluorescing or doubly refractile materials.

The zona glomerulosa on the other hand was considerably broadened, its cells appeared histologically normal, and were laden with sudanophilic droplets that were also Schiff positive (Fig. 7). This zone was also strikingly autofluorescent and contained an abundance of birefrigent material that was evenly distributed as coarse and fine particles (Fig. 8). These observations are in agreement with the description of adrenal cortical changes following hypophysectomy as reported previously by Deane and Greep (1946).

The testes of 3 rats given diet 100 and 3 given diet 150 for 112 days following removal of the pituitary were examined microscopically. In all instances the seminiferous tubules and the Leydig cells were atrophic (Fig. 9). The degree of regression was sufficiently extensive to eliminate any doubt concerning the completeness of removal of the anterior lobe of the hypophysis.

DISCUSSION

Complete surgical removal of the hypophysis is generally believed to result in cessation of growth as manifest by no further increases in body weight and skeletal dimensions. That these growth processes can be reinstituted by the growth hormone prepared from pituitary glands has been conclusively demonstrated. A point arises, however, with respect to tail growth, since Freud, Levie and Kroon (1939) concluded from a series of assay experiments, that (1) tail growth in hypophysectomized rats was more uniformly proportional to the potency of growth hormone preparations than the accompanying increases in body weight and (2) unless the hormone were injected soon after surgical removal of the pituitary, no tail growth was

FIG. 5. Radiograph of the tail of a normal rat from the stock colony. The body weight of this rat was 63 grams at the time of this radiograph.

FIG. 6. Radiograph of the tail of a rat from experiment 1 which had been hypophysectomized at a weight of 62 grams and then maintained for 88 days on ration 100+ 20 per cent Cellu flour at which time it weighed 94 grams. Note the greater tail length and the increase in length and calcification of the individual vertebrae in comparison with the tail of the control animal in figure 5.

FIG. 7. Photomicrograph of a frozen section of an adrenal from a typical animal in experiment 2 sacrificed 112 days after hypophysectomy. Stained with Sudan IV. Note heavy staining of sudanophilic lipids in the zona glomerulosa of the cortex and the almost complete absence of sudanophilic lipids in the zona fasciculata. The cortex as a whole is greatly reduced in width. ×60.

FIG. 8. Photomicrograph of a frozen section of an adrenal from a typical hypophysectomized rat in experiment 2 taken with a polarizing microscope. The birefringent particles are almost entirely confined to the broadened zona glomerulosa. ×60.

FIG. 9. Photomicrograph of a section of testis from a typical hypophysectomized rat in experiment 2. The germinal cells have largely disappeared and the investiture of the tubules is composed mainly of Sertoli cells. Hematoxylin and eosin. ×60.

possible since, according to their findings, cessation of tail growth following hypophysectomy was accompanied by irreversible closure of the epiphyses of a large number of the caudal vertebrae. On the basis of our data, hypophysectomized rats maintained for relatively long periods on purified rations do not show closure of the epiphyses in the caudal vertebrae even when no growth hormone preparation was administered.

During the first 5 weeks of our experiments, the average increases in body weight of all groups of hypophysectomized rats were remarkably similar despite the great differences in the percentage survival within groups. These average increases in body weight fail to reveal an interesting difference which actually existed between the individual growth patterns of rats fed purified diets and those fed laboratory chow. In each of the 8 groups in these experiments, the weight changes of individual rats in the critical period after hypophysectomy could be divided into 3 merging categories: (1) slow but gradual and prolonged weight increases, (2) weight maintenance without appreciable fluctuation and (3) gradual weight loss. Some rats receiving ground chow or chow pellets began to lose weight after hypophysectomy and died in relatively short periods; others maintained their weight for a short interval, followed by weight loss and death. In direct contrast, rats which received either of the purified rations and began to lose weight post-operatively, survived for relatively long periods. Several recovered to the point of weight maintenance and eventually made progressive weight gains. Moreover, rats which maintained their weight in the early post-operative period rarely died and soon began to gain weight slowly and steadily. Lastly, rats which were fed chow diets and showed an early post-operative gain in weight soon ceased to grow and then lost weight for a short period before death. In comparison, hypophysectomized rats provided with purified rations, and which began to gain weight, continued to gain slowly for long periods with practically no mortality within the length of our experimental regimen. Furthermore, whenever unexplainable crises resulted in growth plateaus or weight losses during later stages of the experimental period, those hypophysectomized rats receiving chow invariably had a lesser ability to recover from these recessions than the rats fed purified diets. Thus, a close examination of the changes in body weight of individual rats indicated that a strikingly greater and more prolonged life-sustaining stimulation was elicited in hypophysectomized rats by the purified rations than by the chow diets, especially during periods of stress.

In the experiments of Freud and co-workers, and most other researchers, hypophysectomized rats were provided with food *ad libitum*. Very little information is available concerning food habits and the nutritional requirements of hypophysectomized rats or of the effect of different diets on the general health or body structure of

these animals. In contrast to experiments with voluntary food consumption, Samuels, Reinecke and Bauman (1943) tested the premise that hypophysectomized rats might be able to absorb and metabolize more food than they would voluntarily consume by feeding measured amounts of diet to hypophysectomized rats by stomach tube for periods varying from 15 to 54 days.[3] These animals exhibited definite growth of the skeleton and musculature, as shown by increases in body weight and in femur weight. They also stored nitrogen at a rate about one-third that of intact pair fed controls; nitrogen storage was more rapid than skeletal growth but less rapid than the increases in body weight. On the other hand, fat had been stored at a much higher rate than by intact controls and, probably, accounted for a major portion of the body weight increases. Weights of the adrenals, reproductive organs, thyroids, and spleen decreased in size at rates comparable to those observed in hypophysectomized animals fed *ad libitum*.

It is interesting to compare the caloric intake and growth response of our hypophysectomized rats allowed to eat *ad libitum* with those of Samuels *et al.* which were forced to take food in excess of what they desired. In the latter instance, the caloric intake amounted to about 39.6 calories per rat per day, whereas our animals consumed enough ration 100 and 150 to yield an average of only 22.8 and 22.5 calories per day, respectively, during the first 35 days of the experiment. Towards the end of this period, their weights were in about the same range as the force-fed rats. The average food consumption of our intact rats of the same strain in this weight range has been found to be equivalent to 45 calories on which intake they gained between 4 and 5 grams per day. Thus, the force-fed hypophysectomized rats had to ingest amounts of food almost equivalent to what an intact rat of the same weight would voluntarily eat to attain its maximum increase in body weight. The amount of food fed to these hypophysectomized rats was about 75 per cent in excess of what our hypophysectomized rats of the same weight voluntarily consumed in the process of gaining an average of 0.6 gms. per day. The degree of body weight increase observed in operated rats allowed *ad libitum* access to the purified rations would seem to represent a level more nearly approaching the physiological optimum.

The average protein contents of the carcasses of our hypophysectomized rats fed purified rations were lower than the values for in-

[3] The diet used was composed of a mixture of powdered ovalbumin, lactalbumin, melted butter, corn oil, cornstarch, dextrin, glucose monohydrate, cellu flour, salt mixture, vitamin B-complex, and percomorph oil suspended in water in sufficient amounts that each cubic centimeter of the suspension contained sufficient solids to yield about 3.3 calories upon digestion. The basal composition of this diet was roughly comparable in its carbohydrate, protein and fat distribution to our ration 150 but had 14 per cent of cellu flour in addition. Each day, the hypophysectomized rats were given three feedings of 4 cc. each at 5-hour intervals.

tact rats (Scheer *et al.*, '43) fed comparable diets (15.2 and 14.3 per cent versus 18.7 and 17.9 per cent, respectively). Therefore, it seems probable that the hypophysectomized rats had a slightly lower ability to build protein than the intact rat of the same age. The fact that the protein content of the hypophysectomized rats was maintained at a level so nearly comparable to that of intact rats during a period of growth in which the body weight was slightly more than doubled certainly indicates that a substantial amount of protein synthesis must have occurred. Since carcass analyses were not made at the beginning of the experiment, and since no nitrogen balance studies were made, the actual amount of protein synthesis and the efficiency of protein utilization are not known.

In so far as our data are concerned, there is no evidence that fat metabolism has been altered by hypophysectomy. Unlike force-fed hypophysectomized animals which retained fat at a much more rapid rate than their intact controls, the carcasses of our hypophysectomized rats allowed to feed *ad libitum* did not have a higher fat content than intact rats fed the same diets. This was true even where 45 per cent of the caloric value of the ration was supplied from fat (ration 150). Since the fat content of these animals did not differ significantly from those receiving ration 100, it can be concluded that fats and carbohydrates are utilized as energy sources by the hypophysectomized rats with comparable efficiency.

The values for ash and carbohydrate in the carcasses of the hypophysectomized rats were slightly higher than for intact rats fed comparable rations. It is impossible on the basis of present data to determine whether there is anything of significance about these small differences.

These investigations represent only a beginning in the understanding of the relationship of diet to the survival, growth and body composition of hypophysectomized animals. The unexpectedness of substantial increases in body weight accompanied by tail growth in hypophysectomized rats allowed to eat *ad libitum* made it necessary to obtain data beyond the original purpose of this investigation. Our data needs to be supplemented by carcass analyses of hypophysectomized rats at the time of operation, and after a short period on chow, by determinations of the basal metabolic rate, and by the rate of utilization of the various major food constituents.

SUMMARY

Between 60 and 70 per cent of rats fed purified diets *ad libitum* after surgical removal of the hypophysis survived for experimental periods of at least 88 and 112 days. Only 10 per cent of comparable rats offered laboratory chow survived beyond 45 days and none more than 63 days. The long survival of hypophysectomized rats fed the purified rations was not due to the fine particle size of the purified

rations nor to their lack of undigestible crude fiber. Those hypophysectomized rats which survived for long periods increased considerably in body weight and, apparently, synthesized considerable protein during the growth process. Tail growth was observed, individual caudal vertebrae lengthened, and the epiphyses of the caudal vertebrae did not close. Hypophysectomized rats which had been maintained on purified rations for 112 days, during which time they had doubled their body weight, had a slightly lower protein content but, otherwise, did not differ greatly in their carcass analyses from intact rats fed purified rations for comparable periods.

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MODE OF ACTION OF OESTROGENS ON THE CORPUS LUTEUM

E. O. HÖHN* AND J. M. ROBSON

From the Dept. of Pharmacology, Guy's Hospital Medical School

LONDON

IT IS well known that oestrogens can maintain the activity of the corpus luteum in the rabbit, in the absence of the pituitary. That such luteal tissue exerts its normal secretory activity is shown by the fact that in non-pregnant animals it produces its typical effect on the endometrium, i.e. progestational proliferation, and in pregnant animals, it maintains a normal gestation (Westman & Jacobson, 1937; Robson, 1937, 1939).

The question arises whether the oestrogens act directly on the luteal cells or whether there is some intermediate mechanism which ultimately results in the effect on the corpus luteum. There is indeed other evidence that oestrogens can exert an action on the ovary (Aron & Aron, 1946) and the results of Bullough (1943) suggest that this effect is a direct one, involving action on the ovarian cells by the oestrogen present in the follicular fluid.

The present experiments were started in the expectation that clear evidence for a direct action of oestrogen on the corpus luteum would be obtained, but in this we have been disappointed. On the contrary, the results are in favour of the view that the effect is indirect. It is possible that this is due to technical deficiencies in our methods, and our data are presented in the hope that they may be of help in other and perhaps more conclusive investigations.

METHOD

In order to determine the site of action of oestrogens on the corpus luteum, implants of oestrogens were made into a single corpus luteum of pseudo-pregnant rabbits. The animals were made pseudo-pregnant by the injection of pregnancy urine gonadotrophin. Implantations were carried out using "crystal guns" of several sizes depending on the dimensions of the implant. A crystal gun consists of a fine pointed glass tube into which the implant is inserted. The corpus luteum was pierced with the point of the tube and the crystal driven in by means of a well-fitting central rod used as a piston. The animals were hypophysectomized by the method of Firor (1933) on the day after implantation. Macroscopic observations of the condition of the corpora lutea in the two ovaries were made subsequently at one or

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* Present address: Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta, Canada.

more laparotomies. The animals were finally killed and the two ovaries examined microscopically.

If the implanted oestrogen acts directly on the cells of the corpus luteum it would be expected that shortly after hypophysectomy (about the 4th day) and for some time subsequently, the implanted corpus luteum would be maintained in size and histological development. The remaining corpora lutea of the implanted ovary might show *some* degree of maintenance while the corpora lutea of the unimplanted (control) ovary would be expected to show involution. If, on the other hand, the maintenance of the corpus luteum produced by oestrogen administration is due to an indirect mechanism, e.g. possibly as the result of the formation of another substance acting on the corpus luteum but produced in some other tissue under the influence of the oestrogen, then oestrogen implantation would lead either to some degree of maintenance of the corpora lutea of both ovaries (with adequate dosage) or to failure of maintenance in both ovaries if the dose were inadequate.

RESULTS

Preliminary Experiments on Mice

As a first step, preliminary experiments were made to determine, for certain oestrogens, a dose small enough to be readily implanted or injected into the rabbit's corpus luteum, which would yet exert an oestrogenic effect for at least 10 days.

The oestrogens used were administered to ovariectomized mice, by subcutaneous injection, subcutaneous implantation or intrarenal implantation of solid material; using the crystal gun in the last two instances. The oestrogenic effect was followed by daily vaginal smears. The condition of the smear was noted by the semi-quantitative

TABLE 1. VAGINAL EFFECT OF CERTAIN OESTROGENS IN OVARIECTOMIZED MICE, WITH DIFFERENT METHODS OF ADMINISTRATION

Compound	Mode of administration	Dose	No. of animals tested	Duration of response*	
				Full oestrus, days	Positive responce, days
Triphenyl chlorethylene crystals (obtained by slow evaporation of a benzene solution).	Subcutaneous implantation	0.03–0.49 mg.	7	0–5	
Triphenyl chlorethylene crystals (obtained by slow evaporation of a benzene solution).	Intrarenal implantation	0.024–0.52 mg.	8	0	
1% aqueous suspension of oestrone.	Subcutaneous injection	10–50 μg.	8	0–13	
1% aqueous suspension of oestrone.	Intrarenal injection	10–50 μg.	10	0–4	
Oestradiol dipropionate crystals (obtained by cooling a solution in hot alcohol).	Subcutaneous implantation	16–137 μg.	12	24–55	
Oestradiol dipropionate crystals (obtained by cooling a solution in hot alcohol).	Intrarenal implantation	14–58 μg.	6	9–45	
Stilboestrol crystals (obtained by cooling a solution in hot alcohol)	Subcutaneous implantation	5–50 μg.	10	6–30	
Stilboestrol crystals (obtained by cooling a solution in hot alcohol)	Intrarenal implantation	5–50 μg.	10	5–66	
1% oestradiol dipropionate in cholesterol mixture.	Subcutaneous implantation	20–60 μg.	8	0–8	0–10
0.1% oestradiol dipropionate in cholesterol mixture.	Subcutaneous implantation	10–60 μg.	8	0	0–7

* I.e., the interval between implantation and last day on which the vaginal smear showed complete cornification (full oestrus) or showed partial change (positive response).

method previously described (Robson, 1938) and a smear was judged to be positive as long as it differed significantly from that found in untreated ovariectomized mice. These preliminaries seemed to be essential, since administration of an oestrogen which produces a significant effect in the vagina in the mouse might, when administered into the corpus luteum, be expected to discharge oestrogen into that organ for at least the same period of time.

Renal implantation was used since the corpus luteum is a very vascular structure and it seemed possible that absorption from it might be at a different rate from that which obtains in the comparatively avascular subcutaneous tissues of the mouse. The results are given in Table 1.

It will be seen that oestradiol dipropionate and stilboestrol appeared to be satisfactory, while injections of aqueous oestrone suspension produced effects which were too limited in duration. It should be noted that in experiments with oestrone and triphenyl chlorethylene, intrarenal administration was relatively ineffective as compared to subcutaneous administration (Table 1); while in the case of oestradiol and stilboestrol, comparable effects were produced by both methods of administration. This suggests that oestrone and triphenyl chlorethylene are selectively destroyed or excreted in the kidney, but further investigations on this point are necessary.

As it is known that absorption of steroid hormones is delayed when they are administered as mixtures with cholesterol, experiments were also carried out with implants containing 1% and 0.1% of oestradiol fused with cholesterol. An attempt was made to find a dose which would produce an effect on the vagina less than that of complete oestrus, since it seemed likely that such a dose might produce a very localized effect on the corpus luteum.

Table 1 shows that this result was sometimes achieved with the smaller doeses. The results are expressed as the interval, in days, between implantation and the last day on which full cornification (full oestrus) was observed and also as the interval, in days, up to the last day during which a definite but partial effect, short of complete cornification was observed (positive response).

Experiments on Rabbits

Oestradiol dipropionate implants: (see Table 2) Eight complete experiments with this substance in doses of 5, 10 and 18 μg. were carried out. Experiments thus reported as complete (here, as well as below) are those in which (1) macroscopic examination of the sella turcica at post mortem showed that hypophysectomy had been complete and (2) the period of survival of the animal after the operation was adequate for the purposes of the experiment, as outlined above.

The results of these experiments are summarized in Table 2. It will be observed that they fall into three groups. In the first group, in

one animal only, the corpora lutea were not maintained to the end of the experiment. The results are interesting as they show that the corpora lutea degenerated at about an equal rate in both ovaries. On the 10th day after hypophysectomy examination of the corpora lutea in both ovaries, examined at laparotomy, showed full maintenance; at the 16th day macroscopic examination suggested that some degeneration was already occurring and affecting both ovaries equally. When the animal was killed on the 25th day, the corpora lutea in both ovaries were fully degenerated. In the second group, there was partial maintenance of corpora lutea in both ovaries. This

TABLE 2. EFFECT OF OESTROGEN IMPLANTS INTO A SINGLE CORPUS LUTEUM OF RABBITS HYPOPHYSECTOMIZED ONE DAY AFTER IMPLANTATION

Substance and dose implanted (μg.)	Interval between hypophysectomy and death	Degree of maintenance of the corpora lutea
Oestradial Dipropionate		
5	16	Full in both ovaries
5	6	Partial in both ovaries
10	15	Full in both ovaries
10	25	None in either ovary
10	11	Partial in both ovaries
10	23	Full in both ovaries
18	7	Full in both ovaries
18	7	Full in both ovaries
Stilbestrol		
5	4	Partial in both ovaries
5	6	Full in both ovaries
15	6	None in either ovary
15	7	Full in both ovaries
15	4	Partial in both ovaries
Oestradiol 1% in Cholesterol		
60	5	Partial in both ovaries
60	7	Full in both ovaries
60	7	Full in both ovaries

was observed in two animals 6 and 11 days after hypophysectomy respectively. In these, the corpora lutea had undergone some shrinking and there was some invasion by leucocytes. In the third group, there was full maintenance of the corpora lutea in both ovaries. This was observed in five cases, for periods ranging from 7 to 23 days after hypophysectomy.

Stilboestrol Implants (see Table 2): Five complete experiments with doses of 5 or 15 μg. of the crystalline substance were carried out. In two cases, corpora lutea were fully maintained in both ovaries 6 and 7 days after hypophysectomy respectively. In two cases, partial maintenance was observed in the two ovaries 4 days after hypophysectomy. One case showed failure of maintenance 6 days after hypophysectomy.

Implants of 1% Mixture of Oestradiol Dipropionate in Cholesterol:

Three experiments using 60 μg. of the mixture were completed (see Table 2). In two animals, full maintenance of the corpora lutea was observed on the 7th day after hypophysectomy. In one case, partial maintenance only was shown 5 days after hypophysectomy.

It will be noted that no selective effect on the corpora lutea of the implanted ovary was obtained in any of these experiments. Even when the maintenance effect was incomplete, the corpora lutea of both ovaries were preserved to the same extent. Hence the fact that a selective effect of the oestrogen was not observed, cannot be explained on the assumption that the dose necessary to produce a local effect only is very critical i.e. that a dose little above it will maintain the corpora lutea in both ovaries equally, while a dose little below it will not maintain any corpoa lutea.

DISCUSSION

The above results indicate that under the condition of the experiments, the corpus luteum, with oestrogen implanted in it, was maintained to the same extent as the corpora in the control ovary. In most cases, the corpora of both ovaries were maintained equally; slight differences in luteal development in the two ovaries were clearly not significant. In a minority of cases the results fell into equal groups, showing (a) partial maintenance of the corpora lutea in both ovaries or (b) complete failure of maintenance of the corpora at the time of conclusion of the experiment.

Although the problem cannot be regarded as settled, these results suggest that the maintenance effect of oestrogen on the corpora lutea of hypophysectomized animals is an indirect one. This presents certain difficulties in view of the results of previous work (Bullough, 1943). Nevertheless, the present findings are compatible with the view that oestrogens act directly (and as such) on the cells of the corpus luteum, only if it is accepted that oestrogen locally administered does not diffuse into the cells of the corpus luteum from the site of implantation but is entirely absorbed, at or in the immediate vicinity of this site, directly into the circulation. The highly vascular nature of the organ certainly lends some support to this suggestion, though it must be remembered that local effects have been produced in other highly vascular organs, e.g. by the implantation of androgen into the testis (Dvoskin, 1944; Smith, 1944) and of thyroid stimulating substance into the thyroid (Aron & Aron, 1947).

It should be added that in an attempt to find a decisive answer to the problem, certain other experiments were devised. Transplants of the ovary into the spleen were made in a few animals, but attempts to produce luteinization of the follicles of the transplanted ovaries failed. If the technical difficulties of this experiment could be surmounted, the effect of the implanted oestrogen could be confined to the implanted ovary, as any oestrogen absorbed into the blood stream would

now enter the portal circulation and be inactivated in the liver. Hence if maintenance of the corpora lutea in the implanted ovary under such conditions could be obtained, the response would not be attributable to the effect of circulating oestrogens.

SUMMARY

Small doses of various oestrogens were implanted as crystals or injected as aqueous suspensions into mice to find those which would produce a prolonged oestrogenic effect in amounts suitable for introduction into a rabbit's corpus luteum.

Crystals of suitable oestrogens were implanted into one corpus luteum of pseudo-pregnant rabbits and the animals were then hypophysectomized. In no animals were the implanted corpora maintained selectively. The corpora lutea in both ovaries were either equally maintained, or degenerated at about the same rate. This evidence suggests that oestrogens do not act directly on the luteal cells but through some indirect mechanism.

ACKNOWLEDGMENTS

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QUANTITATIVE BIO-ASSAY OF CHORIONIC GONADOTROPHIN WITH THE MALE FROG

ARTHUR L. HASKINS, JR.[1] AND ALFRED I. SHERMAN[2]

From the Department of Obstetrics and Gynecology, Washington University, School of Medicine

ST. LOUIS, MISSOURI

INTRODUCTION

THE efficiency and accuracy of "pregnancy testing" with the male Batrachia is well established and has been reviewed recently by Galli-Mainini (1948). While adapting the male Rana pipiens at this laboratory for the routine analysis of serum and urine for gonadotrophin, it became expedient to explore the quantitative aspects of the test. Studies were initiated utilizing the conventional minimal effective dose method of bio-assay.

During this procedure it was noted that the time necessary for the appearance of spermatazoa in the test animals' urine was indirectly proportional to the amount of gonadotrophin administered. The time response-dose relationship proved to have a degree of constancy sufficiently great to provide a relatively accurate method of bio-assay. The time response-dose relationship and the minimal effective dose findings are presented as a rapid and efficient quantitative bio-assay method for chorionic gonadotrophin.

It should be noted that the "male frog technique" used in this laboratory differs slightly from that generally in use (Robbins and Parker, 1948). The usual dorsal or ventral lymph sac injection has been replaced by intraperitoneal injection, since there is less escape of the injected solution when the latter route is used. A further revision has been the adoption of blood serum as the test material almost to the complete exclusion of urine. This change was effected because of the higher gonadotrophic titre of serum and because of the lesser toxicity of serum.

EXPERIMENTAL

The male Rana pipiens used in this study varied in weight from 28 to 50 gms. They were all obtained from a single biological supply house from whom a continuous fresh supply could be assured. The chorionic gonadotrophin[3] was prepared commercially and had pre-

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[1] United States Public Health Service Fellow in Obstetrics and Gynecology.

[2] Research Fellow in Obstetrics and Gynecology.

[3] The gonadotrophin was supplied by Winthrop Chemical Company.

viously been standardized against the international standard. To each frog was administered a constant volume of injectate despite the fact that preliminary work had indicated that the injected volume could vary from 1 to 3 mls. without perceptible variation in the response.

Each frog was placed in a separate container following the intraperitoneal injection of 1 ml. of an aqueous solution containing from 10 to 100 I.U. of chorionic gonadotrophin. The animals were examined for the presence of urinary sperm at intervals of 2 to 3 minutes until the presence of sperm indicated a positive response. This was continued in the absence of a positive response to 4 hours after injection. No positive response was obtained after 125 minutes following injection.

One hundred and forty-nine frogs were used in this series and at least 8 animals were tested at each dosage level of 100, 90, 80, 70, 60, 50, 45, 42.5, 40, 37.5, 35, 32.5, 30, 27.5, 25, 22.5, 20, and 10 I.U. of chorionic gonadotrophin. The animals tested at each dosage level were divided into two groups and compared statistically.

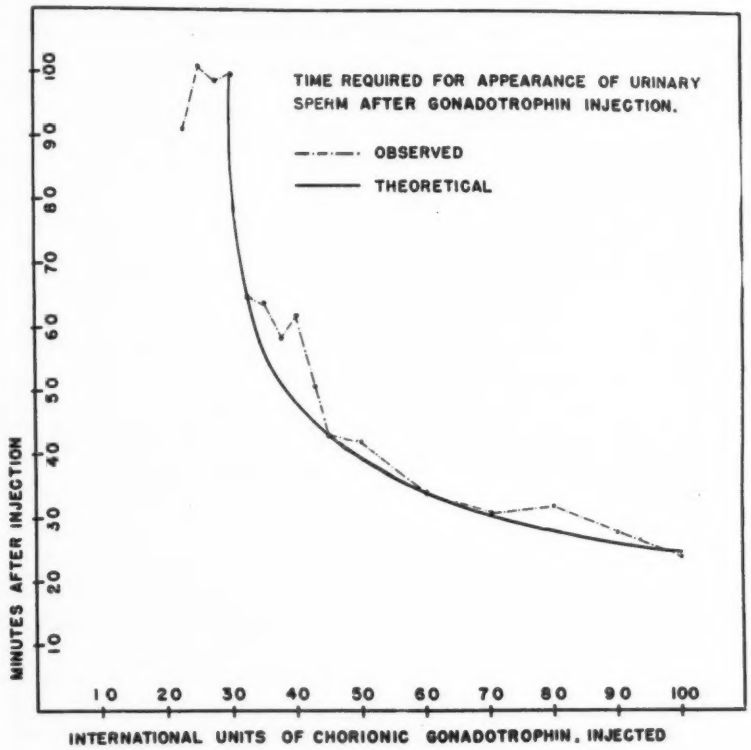

FIG. 1.

RESULTS

The time necessary for a positive response to occur in the test animals was found to vary indirectly and as a hyperbolic function of the size of the dose administered when between 22.5 and 100 I.U. of gonadotrophin was used. At these dosage limits the curve became asymptotic. The mean deviation of the animals at each dosage level was such that 4 animals were requisite to an adequate analysis at any

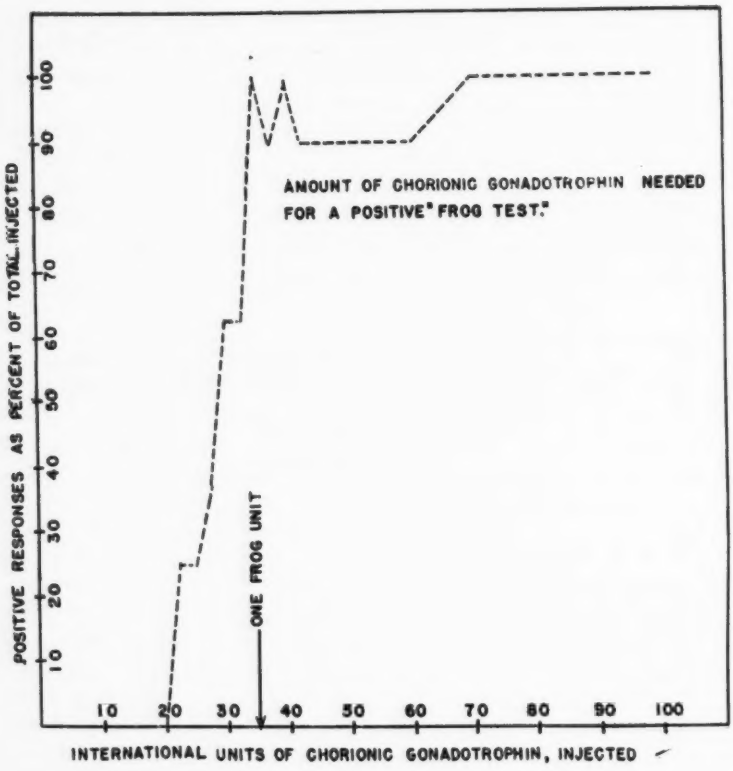

FIG. 2.

given level. An examination of the curve further reveals that at doses between 35 and 60 I.U., estimation of the dose of gonadotrophin from the time required for a positive response is most accurate. Figure 1 is offered as a graphic illustration of these findings.

Figure 2 indicates the response of the test animals to various amounts of intraperitoneally injected chorionic gonadotrophin without regard for the time element, but only in terms of positiveness or negativeness. At 70 I.U. and above, 100% of the test animals yielded

urinary sperm. From 35 to 60 I.U. inclusive, the animals responded with only an occasional negative result. The occasional negative response found in these dosages illustrates the necessity of utilizing two animals in routine "pregnancy testing" and the resultant increase in accuracy as compared to those tests that are made using only one test animal.

Below 35 I.U. of gonadotrophin the percentage of positive responses rapidly decreased and at the level of 20 I.U. and below, the presence of urinary sperm could no longer be detected. As a result of these findings, a male Rana pipiens "frog unit" of gonadotrophin is defined for use in this laboratory as the smallest amount of chorionic gonadotrophic substance necessary to cause a positive response in 3 of 4 test animals. This unit approximates 35 I.U. of chorionic gonadotrophin, which compares favorably with the minimal effective dose of gonadotrophin for a positive response in the male Bufo arenarum Hensel of 40 I.U. (Galli-Mainini, 1948).

When determining gonadotrophic titre in this laboratory an attempt is made to locate two points on the time response-dose curve, Figure 1, by dilution of the test substance and repeated testing with groups of 4 frogs. Calculation then gives the gonadotrophic titre of the substance tested expressed as I.U. or frog units. The estimated titre may further be verified, if desired, by calculating the amount of the test substance equivalent to 35 I.U. and testing of an appropriate number of animals at this level and below for the minimal effective dose.

SUMMARY

The male Rana pipiens reacts to chorionic gonadotrophin by liberating sperm into the urine. At least 35 I.U. of gonadotrophin are necessary to provide a positive response in 75% of these animals, whereas doses of 70 I.U. and above initiate a positive response in 100% of tested animals. The time required for the liberation of sperm is indirectly proportional to the dose of gonadotrophin administered. The time response-dose relationship is constant enough to provide a relatively accurate quantitative bio-assay technique which is complemental to the conventional minimal effective dose technique.

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COMPARATIVE ACTIVITY OF THIOURACIL AND OTHER ANTITHYROID COMPOUNDS IN THE RHESUS MONKEY

D. A. McGINTY AND M. L. WILSON
From the Research Laboratories, Parke, Davis and Company
DETROIT, MICHIGAN

INTRODUCTION

THE selective iodine concentrating mechanism of the thyroid gland has been clearly demonstrated to occur in two independent steps (Vander Laan and Vander Laan, 1947) (Taurog, Chaikoff and Feller, 1947). The first step which involves absorption of iodide ion from the blood is not inhibited by antithyroid agents of the thiouracil type and accounts for but a small portion of the total iodine present in the gland at any time. The second step involves the conversion of absorbed iodide into organically bound form, a considerable portion of which is of hormonal character (Wolff and Chaikoff, 1947). This mechanism accounts for the greater portion of accumulated iodine of the normal gland and is inhibited by thiouracil-like antithyroid drugs. On the basis of these considerations, McGinty and collaborators (1948) quantitated the activity of several antithyroid agents in rats and chicks following a single subcutaneous injection of the compounds in varying doses. The duration of inhibition of hormone synthesis as evidenced by block to iodine collection was determined by administration of tracer doses of radioactive iodine at intervals following administration of drug, iodine absorption being followed by radioactivity counts on excised glands. In the rat, the antithyroid potency of the compounds studied paralleled in a general way the activity of the same substances as estimated by the 10 day chronic feeding test (Astwood, Bissell and Hughes, 1945; McGinty and Bywater, 1945). In the chick this parallelism did not hold. The duration of action of minimal effective doses in both rat and chick was not materially different, escape from the blocking action occurring at 8 hours in the rat and 6 hours in the chick.

Using the same general principle, Stanley and Astwood (1947) assayed 32 compounds for antithyroid activity in man. Following administration of radioactive iodine, radioactivity counts were recorded with a shielded G-M counting tube placed over the sternal notch until such time that an individually characteristic "accumulation gradient" or rate of iodine uptake by the thyroid was established. At this time,

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usually from one to two hours after radioiodine administration, varying amounts of the compounds under study were administered orally. Potency was estimated from the degree and duration of alteration of the accumulation gradient slope.

This important investigation brought out two facts, one, that the antithyroid activities of the compounds evaluated in man were in most instances quite different from the potencies as estimated from rat and chick experiments, and, two, that these activities appear to correspond more closely to results obtained by clinical experience in treatment of thyrotoxicosis rather than what would be expected from rat or chick experiments.

It is the purpose of this communication to present similar comparative studies on a few compounds in the monkey. The drugs studied included thiouracil and the 6-methyl, 6-propyl and 6-benzyl derivatives, 2-mercapto-5-amino-thiadiazole (TC-68), 3-(phenylaminomethyl)-2-thiazolidinethione (TC-105), 2-mercaptoimidazole, 2-mercaptoimidazoline, 2-mercaptothiazole and 2-mercaptothiazoline. The last four compounds were of particular interest because of the high antithyroid potencies of 2-mercaptoimidazole and 2-mercaptothiazoline reported by Stanley and Astwood in man—10 and 2.5 times that of thiouracil respectively.

METHOD

One female and nine male rhesus monkeys of 4–7 kilograms body weight were housed singly in roomy screen-bottom cages in an air-conditioned room. They were given a rotated diet, *ad libitum*, of cooked cereals, fresh fruits and vegetables, bread, peanuts and milk. In addition, each animal received 1.2 ml. of a liquid vitamin supplement (Abdec) daily on a sugar cube.

At the outset of each experiment, which was repeated at monthly intervals, carrier-free I^{131} was administered intravenously in sterile saline at doses of approximately 20 microcuries. Radioactivity counts were taken a few minutes following radioiodide injection and at one-half hour intervals for three hours at which time the antithyroid drug under study was administered. Counts were then taken at hourly intervals until the seventh hour and again at the 11th, 24th and 48th hour from radioiodine injection.

Counting was done by means of a 6 cm. diameter shielded Victoreen tube and a Tracerlab Autoscaler. Records were taken over the midline of the neck in the angle between the clavicles and suprasternal notch. The G-M counter was checked at least twice daily against a radiocobalt standard. Antithyroid drugs were administered in gelatin capsules inserted manually into the posterior pharynx.

RESULTS

In conformity with the results reported by Stanley and Astwood for man, we found also that the rate of radioiodine uptake by the thyroid of the monkey as measured by the externally positioned counter, when plotted against time, forms a distorted S-shaped curve,

the greater curvature of which is parabolic in shape. When radioactivity counts are plotted against the square root of the elapsed time in minutes, the major portion of the curve, after the first half hour and for at least 11 hours, was essentially linear. This is shown in Figure 1 in untreated animals in which individual neck counts are plotted against the time at which they were recorded. Repetition of these control experiments indicated that the slope of the iodine col-

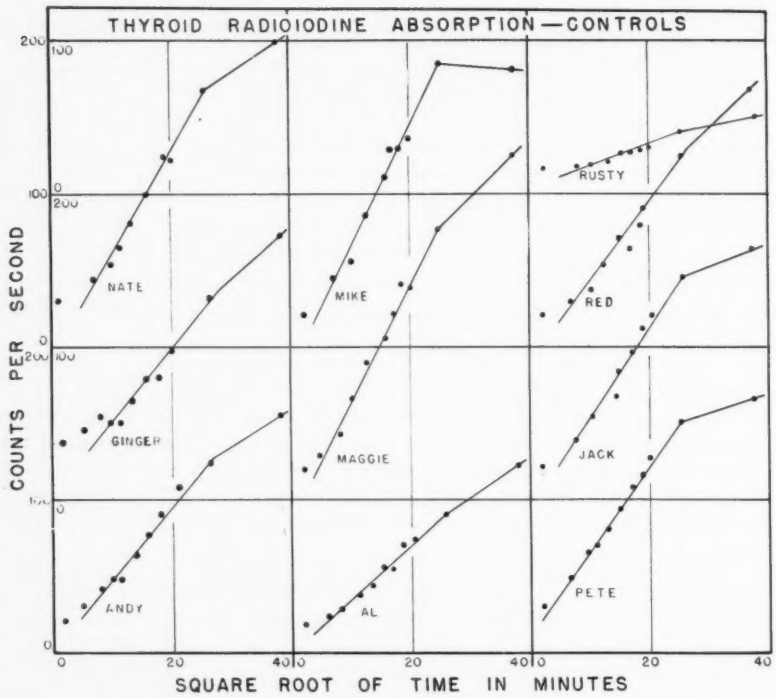

FIG. 1. Showing uptake of radioactive iodine by the thyroid of ten normal Rhesus monkeys following intravenous injection of 20 microcuries carrier-free radioiodide (I^{131}). Radioactivity in the thyroid, taken from Geiger-Mueller counter measurements over the suprasternal notch, is plotted against the square root of the time in minutes from radioiodide injection. The straight line through the individual points on the graph is indicative of the rate of accumulation of iodine by the thyroid over the time interval measured and of the corresponding rate of hormone synthesis.

lection curve varied between different monkeys and in the same monkey at different times. It was necessary, therefore, that estimations of potency of administered drugs be made on deviations from the curve, the slope of which was established immediately preceding administration of the compound under study. Because of the persistently flat curve in the case of Rusty, experiments on this animal were not undertaken.

Experimental results with thiouracil and the three derivatives of thiouracil investigated are shown in Figure 2. The initial rate of iodine collection is plotted as a straight line from neck count data taken at half hour intervals beginning one-half hour after injection of radioiodine and extending to the end of the third hour, when the drug was administered. This slope is extended as a broken line to the 12th

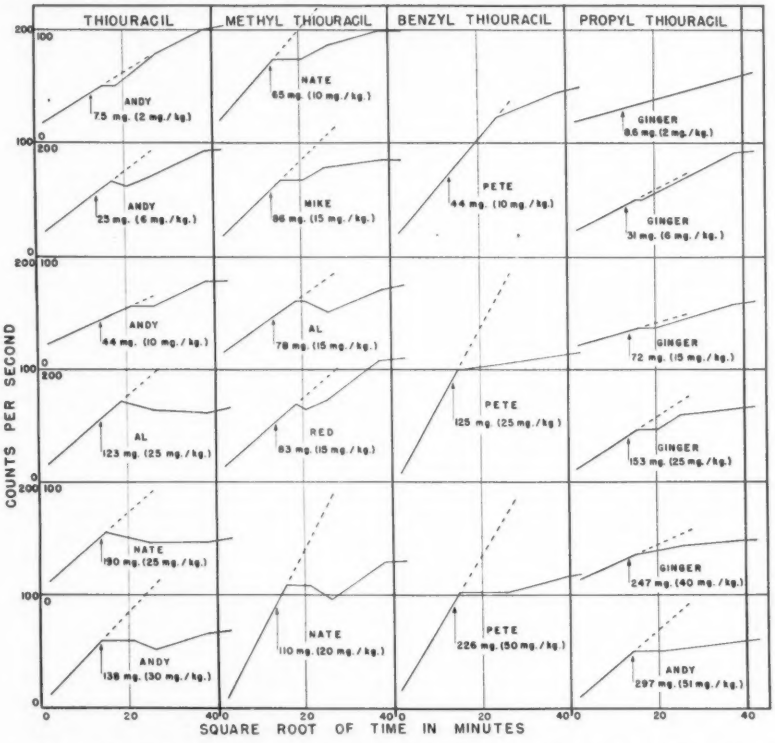
THIOURACIL
METHYL THIOURACIL
BENZYL THIOURACIL
PROPYL THIOURACIL
ANDY 75 mg. (2 mg./kg.)
NATE 65 mg. (10 mg./kg.)
GINGER 86 mg. (2 mg./kg.)
ANDY 23 mg. (6 mg./kg.)
MIKE 86 mg. (15 mg./kg.)
PETE 44 mg. (10 mg./kg.)
GINGER 31 mg. (6 mg./kg.)
ANDY 44 mg. (10 mg./kg.)
AL 78 mg. (15 mg./kg.)
GINGER 72 mg. (15 mg./kg.)
AL 123 mg. (25 mg./kg.)
RED 83 mg. (15 mg./kg.)
PETE 125 mg. (25 mg./kg.)
GINGER 153 mg. (25 mg./kg.)
NATE 190 mg. (25 mg./kg.)
GINGER 247 mg. (40 mg./kg.)
ANDY 138 mg. (30 mg./kg.)
NATE 110 mg. (20 mg./kg.)
PETE 226 mg. (50 mg./kg.)
ANDY 297 mg. (51 mg./kg.)
COUNTS PER SECOND
SQUARE ROOT OF TIME IN MINUTES

FIG. 2. Showing deviations from the established rate of uptake of radioiodine by the thyroids of monkeys when given varying doses of thiouracil and three of its 6-substituted derivatives. The solid portion of the curves represent the accumulation slope of iodine uptake as plotted from radioactivity measurements taken in the first three hour period following radioiodide injection and after oral administration of the antithyroid compound at the beginning of the fourth hour. The broken line indicates anticipated radioiodine uptake for an 11 hour period had no drug been given. Full antithyroid activity is indicated by no increase in radioactivity over the thyroid region.

hour ($t^{\frac{1}{2}}_{min} = 25.5$) to indicate expected radioiodine absorption had no drug been given.

A dose of 7.5 mg. thiouracil, equivalent to 2 mg. per kg. body weight, exerted only a brief inhibitory action on iodine uptake, the curve of accumulation of radioiodine by the thyroid resuming a pattern practically the same as that expected from the untreated animal.

Twenty-five milligrams of thiouracil in Andy was more effective and persisted for about two hours when iodine collection was resumed at a somewhat reduced rate. This reduction in rate of radioiodine uptake was a usual occurrence following temporary inhibition and may have been due to loss of radioiodine through the kidneys during the non-absorptive period. A larger dose of 10 mg. per kg. in Andy induced an inhibition of no more than four hours duration. Administration of thiouracil in amounts equivalent to 25 and 30 mg. per kg. in Al, Nate and Andy were fully effective for 24 hours or longer. Any attempt to evaluate the duration of action of the drugs beyond 24 hours from the 48 hour radioactivity count was invalidated by lack of information on the fate of injected radioiodine during the thyroid inhibition period.

Results with methyl thiouracil were variable. Doses of 10 and 15 mg. per kg. were effective for periods varying from one to four hours although, with the exception of Red, some reduction in the rate of radioiodine absorption by the thyroid was evident for 24 hours. Twenty milligrams per kilogram methyl thiouracil was fully inhibitory for 8 hours, following which iodine absorption was resumed at almost the initial rate. The number of experiments with methyl thiouracil were too few to evaluate comparatively the antithyroid activity of this compound with thiouracil, but it does not appear to differ from the latter substance materially. This is consistent with clinical experiences with this drug.

Benzyl thiouracil inhibited iodine collection by the thyroid in two experiments with 25 and 50 mg. per kg. It was wholly inactive at a 10 mg. dose in Pete, the trend of iodine absorption away from the projected gradient being considered of no significance since it occurred 12 hours following administration of the drug. Benzyl thiouracil was estimated by Stanley and Astwood to be 0.75 times as active as thiouracil on the basis of their thyroid radioiodine absorption experiments in man. Monkey experiments, while too few to provide quantitative data, indicate that this compound is at least in the same order of activity as thiouracil.

Propyl thiouracil did not wholly inhibit radioiodine collection in doses up to 40 mg. per kg. body weight. In a single experiment, a dose of 51 mg. per kg. caused an iodine block lasting for 24 hours. These results conform in a general way with those of Stanley and Astwood which indicated that propyl thiouracil was less active than thiouracil.

2-Mercaptoimidazole was reported by Stanley and Astwood to have an antithyroid activity at least 10 times that of thiouracil. On the basis of similar experiments in monkeys, we estimated that this compound was 20 or more times as active as thiouracil. It will be observed from Figure 3, that a dose of 0.9 mg. or 0.2 mg. per kg. in Nate was only slightly inhibitory to radioiodine collection. However, 0.6 mg. per kg. in the same monkey produced a complete block to iodine

uptake for a period of approximately eight hours. In three other experiments, doses of 1.0 mg. per kg. were completely inhibitory for periods up to 24 hours. These results are comparable to those obtained with 25 mg. per kg. of thiouracil.

2-Mercaptoimidazoline, the saturated analog of the highly active 2-mercaptoimidazole, was found to be fully inhibitory to radioiodine

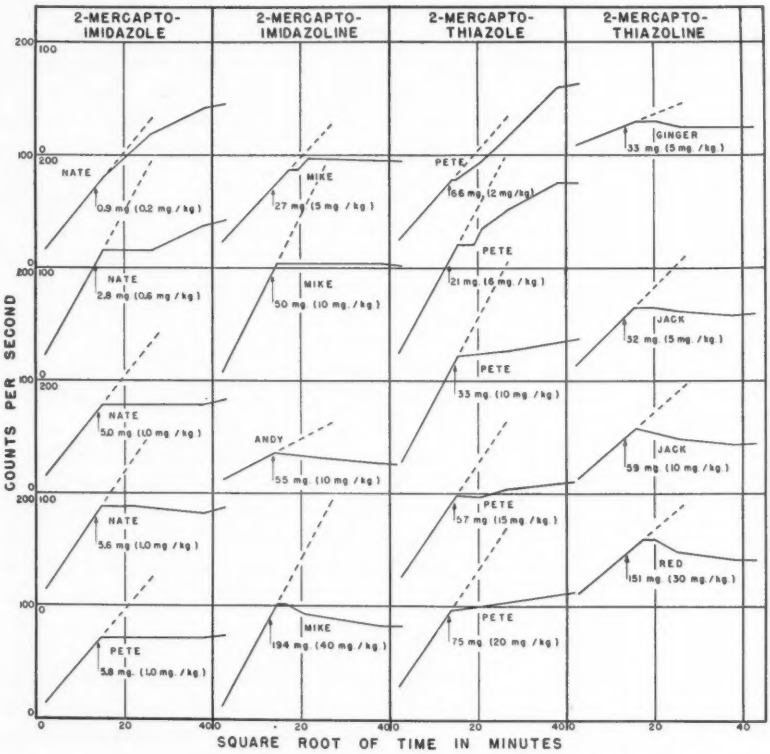

FIG. 3. Showing alterations in the accumulation gradient of radioiodine by the thyroids of monkeys given varying amounts of 2-mercaptoimidazole, 2-mercaptoimidazoline, 2-mercaptothiazole and 2-mercaptothiazoline.

absorption for a period of at least 24 hours in doses of 10 mg. per kg. and suggestive in a single monkey of prolonged inhibition in a dose of 5 mg. per kg. On a comparative basis, this substance was estimated to be at least twice as active as thiouracil, a finding contrary to that reported by Stanley and Astwood. These workers rated 2-mercaptoimidazoline half as active as thiouracil in man.

2-Mercaptothiazole and 2-mercaptothiazoline, the sulfur analogs of 2-mercaptoimidazole and 2-mercaptoimidazoline, were studied in

monkeys. 2-Mercaptothiazoline was estimated by Stanley and Astwood to possess 2.5 times the activity of thiouracil. Our experiments in monkeys were incomplete in that the minimal inhibitory dose of this compound was not determined. Administration of 5 and 10 mg. per kg. blocked iodine collection by the thyroid for 24 hours or more and by comparison was estimated to be more than five times as active as thiouracil, an appraisal higher than the value of 2.5 assigned by the Boston workers. 2-Mercaptothiazoline was evaluated for antithyroid activity by the 10-day chronic feeding test in rats (Astwood, Bissell and Hughes, 1945; McGinty and Bywater, 1945) and results from both laboratories were in agreement that this compound was 1.3 times as active as thiouracil. It is apparent that its activity in the human and in monkeys is comparatively much greater.

On the basis of structural relationship and relative antithyroid activities in both man and monkey of 2-mercaptoimidazole and 2-mercaptoimidazoline on one hand and of 2-mercaptothiazole and 2-mercaptothiazoline on the other, it was expected that 2-mercaptothiazole might possess extremely high antithyroid activity compared with thiouracil. It was found, however, that doses of 10 mg. per kg. were required to maintain the thyroid in an inhibited state for 24 hours. The compound is estimated to be about twice as active as thiouracil.

2-Mercapto-5-aminothiadiazole (TC-68) and 3-(phenylaminomethyl)-2-thiazolidinethione (TC-105), two compounds investigated extensively in rat and chick experiments, were found in monkeys to be approximately twice as active as thiouracil. In the case of TC-68, this value agreed with that reported by Stanley and Astwood. These investigators did not test TC-105.

DISCUSSION

Estimations of antithyroid potencies of various compounds on the basis of inhibition of radioiodine collection by the thyroid after injection of carrier-free radioiodide appear valid. Any significant absorption of iodine by the thyroid from blood whose iodide concentration has not been altered materially may be considered as a measure of and directly proportional to hormonal iodine formation by thyroid cells. Thus, any reduction in the rate of absorption of iodide whether measured directly or by the tracer technic may be considered due to decreased hormone synthesis and a quantitative measure of antithyroid potency of most substances whose administration produces this effect. In this connection, it must be pointed out that Vander Laan and Vander Laan (1947) have shown that the goitrogenic action of KSCN is due not to inhibition of protein binding of iodine and hormone synthesis but to interference with entry of iodide into thyroid cells.

Stanley and Astwood call attention to certain limitations in the radioiodine absorption method as a measure of clinical effectiveness of

certain antithyroid compounds. They point out that the procedure does not distinguish between compounds relatively weak by the test but clinically effective because of cumulative action and those highly active inhibitory agents which may be clinically ineffective because of rapid elimination and non-cumulative properties. The same objection applies to the findings on monkeys. Examination of the curves on propyl thiouracil and benzyl thiouracil, which according to Stanley and Astwood are short-acting antithyroid agents, indicates that these drugs, even in the larger doses, are not completely inhibitory. This may be due to rapid kidney loss, but in human subjects we have shown (McGinty, Sharp, Dill and Rawson, 1949) that propyl and benzyl thiouracil are, in fact, eliminated at the same rate as thiouracil and methyl thiouracil. In contrast, however, to the latter substances, propyl and benzyl thiouracil appear in the urine in acid-hydrolyzable form. We have speculated that this conjugation may account for the clinical ineffectiveness of benzyl thiouracil and to a lesser extent for that of propyl thiouracil relative to the high antithyroid potency of these compounds in rats and chicks. Whether or not conjugation occurs in the monkey, we do not know.

The monkey appears to be considerably less sensitive to antithyroid agents than man. Comparison of available data indicates that 5 to 10 times as much drug is required to produce comparable thyroid inhibitory effects in the monkey.

On the whole, our estimation of relative antithyroid activities from the monkey experiments agreed with those conducted by a similar procedure in human subjects. The only noteworthy exception was in the case of 2-mercaptoimidazoline which we rated as twice as active as thiouracil but to which Stanley and Astwood assigned a value of 0.5. Both laboratories agreed as to the extremely high potency of 2-mercaptoimidazole and it is hoped that further clinical information on this interesting drug will be forthcoming.

It was noted that control accumulation gradient curves from serial experiments in the same animal were invariably less steep than preceding curves. What caused this is not known. Rough calculations from neck counts, estimated geometry, etc. indicated that three-quarters or more of the administered dose of radioactive iodine was taken up by the thyroid within 48 hours. Further crude estimates revealed that this absorption may have amounted to 50 or more microcuries per gram of thyroid tissue. According to the recent extensive work of Haines, *et al.* (1948), this dose of radiation over the whole decay period approaches that which caused definite reduction in mass of thyroid tissue and fall in B.M.R. in thyrotoxic patients.

SUMMARY

Estimations of antithyroid activity of a group of compounds were carried out in normal monkeys after intravenous administration of radioactive iodine by observing the degree and duration of alteration

of an established rate of accumulation pattern by the thyroid gland. The antithyroid drugs were administered orally and radioactivity in the thyroid was recorded by external counting.

Assigning thiouracil an evaluation of 1.0, the following results were obtained: 2-mercaptoimidazole, 20; 2-mercaptothiazoline, >5; 2-mercaptoimidazoline, 2; 2-mercaptothiazole, 2; 2-mercapto-4-aminothiadiazole, 2; 3(phenylaminomethyl)-2-thiazolidinethione, 2; methyl thiouracil, 1; benzyl thiouracil, 1; and propyl thiouracil, 0.5. These values are in general agreement with estimations similarly obtained in man by Stanley and Astwood.

ACKNOWLEDGMENTS

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A NOTE ON EPITHELIAL METAPLASIA IN THE MALE GENITAL TRACT[1]

HOWARD A. BERN

From the Department of Zoology, University of California

BERKELEY, CALIFORNIA

ATTEMPTS have been made by several investigators to study various instances of metaplasia of sex accessory epithelia and to arrive at a possible common etiologic factor for all such metaplasia. On the basis of somewhat inconclusive data, McCullough and Dalldorf (1937) suggested that vitamin A deficiency was a prerequisite for all true epithelial metaplasia. Hume, Burbank, and Korenchevsky (1939) produced identical lesions in male rat sex accessories by deprivation of vitamin A and by administration of large amounts of estrogen. Whether such identical lesions occurred in non-genital tissues was not fully indicated. Cramer (1942) obtained similar results from experiments on the rat uterus.

Kasman and Gold (1933) and Sutton and McDonald (1943) studied metaplasia in the human prostate, and the latter concluded that benign metaplastic changes following transurethral resection were due to "the basic polymorphism of epithelial cells" and their reaction to necrotic and regenerative processes following the resection.

In the course of a series of studies involving the experimental production of sex accessory pathologies in the male Dutch rabbit, several instances of epithelial metaplasia were noted. The effect of short and long treatments with estrogens in inducing a non-keratinized, non-squamous, stratified columnar ("transitionoid") metaplasia has already been reported in detail (Bern, '49a, b). An entirely different type of epithelial metaplasia, hitherto unreported, was observed in a large number of animals employed during the course of these studies. Keratinized nodular lesions were found in the prostate of 45% and in the vesicular gland of 71% of a total of 80 normal and experimental rabbits. In no instance were these lesions found in the seminal vesicle.

The lesions consist of groups of closely packed epithelial cells with large nuclei and undifferentiated cytoplasm and show frequent mitotic figures (figs. 1–2). They originate as dedifferentiated or un-

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differentiated clusters of cells in the alveolar walls in both the prostate and vesicular gland and proliferate under the epithelium into the stroma in much the same way as a neoplastic growth. The cells of the lesion bordering on the lumen of the gland are stratified squamous and show some degree of keratinization, often quite extensive (fig. 2). The adjacent epithelium sometimes undergoes squamous metaplasia and contributes to the keratinization. Occasionally, this latter process is accompanied by leucocytic infiltration.

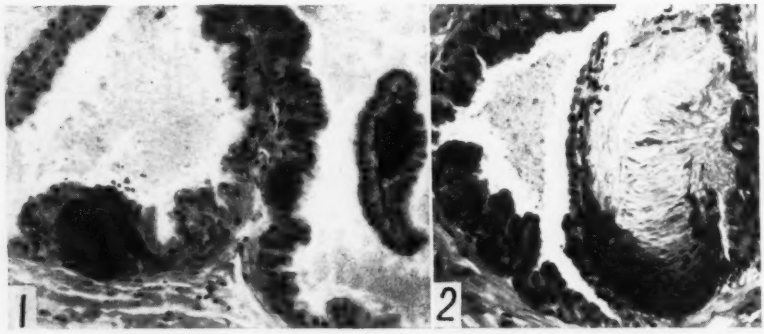

FIG. 1. Two lesions from vesicular gland of untreated rabbit. Lesion on right is compressed between alveolar walls. H. &. E. ×150.
FIG. 2. Keratinized lesion showing involvement of large segment of alveolus in vesicular gland of untreated rabbit. H. & E. ×150.

The lesions are histologically indistinguishable from those described as due to avitaminosis-A in epithelial tissues, including those of the sex accessories, of other animals (Wolbach and Bessey, 1942) and occurred "spontaneously" in our series of Dutch rabbits. Rabbits have need of relatively more vitamin A in their diet than the rat and the pig, and possibly fowl, as was early demonstrated by the experimental production of xerophthalmia (Nelson, Lamb, and Heller, 1922). The histology of avitaminotic-A lesions in the rabbit evidently has not been recorded in the literature, but gross changes indicate similarity to the picture seen in other animals (Wolbach, 1937).

Xerophthalmic indications of avitaminosis-A were incidentally noted in several of our rabbits, along with the low fertility of the breeding stock. Microscopic histologic sections of submaxillary salivary glands and pancreas, as well as other urogenital organs, have been made from time to time, however, and except for rare epididymidal keratinization reported elsewhere (Bern, 1949b), no other histologic evidence of possible vitamin A deficiency has been found in any of the animals examined. However, the recent work of Wilson and Warkany (1947) has indicated the special sensitivity of urogenital

sinus derivatives (including the prostate complex) to lack of vitamin A in the embryonic rat.

To test the adequacy of our rabbit food pellets (a standard commercial "complete" diet), spectrophotometric analyses for vitamin A and carotene content were carried out, and 0.0012–0.0014% carotene was found. The true biologic potency is probably considerably less. Cod liver oil with high vitamin A content was administered to determine if the occurrence of the lesions could be prevented. Of 6 mature rabbits receiving about 10 ml. of oil poured over their food pellets once a week for 2 to 5½ months, three individuals showed lesions regardless of duration of oil administration. Another series of 9 immature (three months of age) male Dutch rabbits was divided into two groups (separating littermates whenever possible): one group of 4 received one ml. of cod liver oil orally by means of a syringe three times weekly for 22 weeks; the second group of 5 served as controls and received the standard diet without cod liver oil. All 9 rabbits upon histologic examination after sacrifice showed lesions in their vesicular glands, although none were seen in the prostates (only sample sections of each gland were prepared).

No conclusions can be drawn as to the adequacy of vitamin A and its precursors in the diet. However, cod liver oil containing vitamin A is evidently unable to prevent or alleviate these lesions in the dosages employed. The cause of the spontaneous lesions in the rabbit, thus, remains undetermined.

In addition to the stratified columnar metaplasia occurring in virtually all male rabbit sex accessories after prolonged estrogenization and the keratinized, stratified squamous spontaneous lesions described above, several other instances of epithelial metaplasia have been noted. These include:

(1) Squamous metaplasia with near-cornified surface cells (due to compression) of vesicular gland epithelium in contact with surgical sutures, distinct from the keratinized nodular lesions; may also result from early contact with methylcholanthrene or paraffin pellets.

(2) Stratified columnar metaplasia of seminal vesicle surface epithelium in contact with voluminous blood clots.

(3) Stratified columnar metaplasia of prostate epithelium due to post-operative infection; accompanying atrophy.

The distinct differences between the spontaneous pathologic lesions (keratinized nodules) and the other types of metaplasia observed lead us to believe that not all metaplasia in the male rabbit urogenital tract is due to the same basic etiologic factor. The prostate complex obviously has at least temporary ability to produce keratinized squamous metaplastic epithelium, but does not so respond under the influence of estrogen. It is possible, however, that the keratinized nodular lesions are established in the younger tissues and that this special reactivity is lost with age.

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THE EFFECT OF ADRENAL CORTEX EXTRACT WITH AND WITHOUT EPINEPHRINE UPON THE WORK OF ADRENALLY INSUFFICIENT RATS

DWIGHT J. INGLE, AND JAMES E. NEZAMIS
From the Research Laboratories, The Upjohn Company
KALAMAZOO, MICHIGAN

EPINEPHRINE has the property of temporarily increasing the height of contraction of "fatigued" skeletal muscle. Radwanska (1910) found that the administration of epinephrine to the "fatigued" adrenalectomized animal gives a temporary increase in work output. Although the hormones of the adrenal cortex are of primary importance in restoring the ability of the adrenalectomized animal to work (Hartman, Brownell and Lockwood, 1932), the question of the importance of the adrenal medulla and its hormone epinephrine in the maintenance of vigor and resistance has remained unanswered. Ingle and Lukens (1941) found that the work performance of the adrenal demedullated rat was depressed below that of normal animals during the first few hours of work and that the administration of epinephrine with glucose would temporarily restore to normal the work rate of "fatigued" adrenalectomized rats. One of us (D.J.I., unpublished) has observed that the continuous subcutaneous injection of adrenal cortex extract with epinephrine to adrenalectomized rats in collapse and near death can cause a reversal of symptoms and recovery which are not obtainable with adrenal cortex extract alone. Recant *et al.* (in press) have observed a synergistic effect of epinephrine and adrenal cortex extract in suppressing the eosinophil count in patients with Addison's disease. It has remained difficult to restore either adrenally insufficient animals or patients to full normal vigor by the administration of cortical extracts and steroids. The possibility that removal or destruction of the adrenal glands creates a deficiency of epinephrine or other principles of the adrenal glands which may be required for normal vigor has been only partially tested by the present studies.

In this study we have tested the possibility that the work performance of the adrenally insufficient rat can be improved to a greater extent by the administration of epinephrine and adrenal cortex extract than by the administration of an equal amount of adrenal cortex extract alone. The results were negative.

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METHODS

Male rats of the Sprague-Dawley strain which weighed 200 ± 2 grams were used. The diet was Archer Dog Pellets. The procedures for the stimulation of muscle were according to Ingle (1944) with the following modifications. A Nerve Stimulator, Model B. Upjohn, was used to stimulate muscle at the rate of five times per second by a DC pulse of 20 milliamperes having a duration of 20 milliseconds. An electrode was placed on the lower tibia of the left back leg and the second electrode on the contralateral back foot, thereby activating all of the musculature of both hind legs. The gastrocnemius muscle of the left hind leg was weighted with 100 grams. The distance that the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 gram-centimeters of work.

A solution of 0.9 per cent sodium chloride containing 1 mgm. of ascorbic acid per cc. and varying concentrations of adrenal cortex extract (ACE) and epinephrine was infused into the jugular vein by means of a constant injection apparatus which delivered fluid from each of 12 syringes at the rate of 20 cc. in 24 hours. In Experiments 2 and 3, glucose (C. P. Merck) was added to the infusion solution in amounts of 200 mgm. per 100 grams of rat per hour. This load of glucose prevented the hypoglycemia which otherwise developed in the working rats. The adrenal cortex extract represented 40 grams of beef adrenal glands per cc. and was free from alcohol. The ascorbic acid acted as an anti-oxidant which effectively preserved the epinephrine.

Our standard procedure for the intravenous administration of solutions was to insert a short bevel 22-gauge hypodermic needle through the pectoral muscle into the jugular vein in a cephalad direction. This procedure was used in Experiments 1 and 2. No complications developed until hypertonic solutions of glucose were injected. The administration of solutions containing glucose in Experiment 2 resulted in some thrombosis of the vein in almost every rat and occlusion of the vein and leakage into the tissues in approximately 25 per cent of the animals. The results from animals in which leakage occurred were discarded. These complications occurred with equal frequency in rats with and without epinephrine. In Experiment 3, a small plastic tube was inserted in the jugular vein and directed towards the heart. This procedure did not cause occlusion and leakage at the point where the infusion fluid entered the vein from the catheter but there occurred respiratory distress and sharp decreases in the rate of work more frequently in the control animals than in animals which received epinephrine. In some instances thrombi were found occluding major vessels.

The animals were anesthetized with phenobarbital sodium and cyclopal sodium. Adrenal enucleations were performed by the procedure of Ingle and Nezamis (in press) and adrenalectomies by the procedure of Ingle and Griffith (1942). The animals were subjected to the work test immediately following the operation. Stimulation was continued until the muscle ceased to respond or for a maximum of 24 hours. Temperature was constant at $28 \pm 0.5°C$.

EXPERIMENTS AND RESULTS

One rat of each pair received 20 cc. of a solution containing epinephrine and the control animal received a solution without epinephrine.

In Experiment 1 (Table 1), adrenal enucleated rats were given ACE with and without epinephrine. Five groups of rats were given 4 cc. of ACE per 24 hours. The addition of epinephrine in amounts of 2, 5, 10, and 20 parts per million did not significantly improve work performance and 40 parts per million suppressed work performance. At this dose of ACE, 61 of 66 rats which received epinephrine died before the end of 24 hours and 53 of the 66 rats without epinephrine died.

TABLE 1. THE WORK PERFORMANCE OF ADRENAL ENUCLEATED RATS GIVEN ADRENAL CORTEX EXTRACT WITH AND WITHOUT EPINEPHRINE

ACE cc. 24 hrs.	Ephinephrine concentration	Number of pairs	Total recorder revolutions. Averages and range	
			Epinephrine	Controls
4	1:500,000	12	11507 (5584–16174)	13641 (6796–35235)
4	1:200,000	12	11682 (6880–16229)	10813 (6678–19635)
4	1:100,000	12	13825 (5593–28129)	12622 (6092–23527)
4	1: 50,000	18	10526 (5295–18797)	13273 (5047–25994)
4	1: 25,000	12	9566 (4858–15808)	14239 (4011–24555)
8	1:500,000	12	20119 (12242–28138)	18222 (8047–22836)
8	1:100,000	12	20514 (7007–30028)	22518 (16780–27286)
8	1: 50,000	12	9880 (6198–16295)	18393 (6727–35638)
15	1:500,000	12	21804 (7704–37764)	21354 (11275–32303)
15	1:100,000	12	17313 (7815–30526)	24769 (8620–36004)
15	1: 50,000	12	20154 (5506–37917)	21753 (5756–38800)

Three groups of rats were given 8 cc. of ACE per 24 hours. The addition of 2 and 10 parts of epinephrine per million did not significantly change work performance but 20 parts per million definitely suppressed work. At this dose of ACE, 22 of 36 rats which received epinephrine died during 24 hours and 8 of 36 rats without epinephrine died.

Three groups of rats were given 15 cc. of ACE per 24 hours. The addition of 2, 10, and 20 parts per million of epinephrine failed to improve work performance. At this dose of ACE, 24 of 39 rats which received epinephrine died during 24 hours and 10 of 39 rats without epinephrine died.

In Experiment 2 (Table 2), all of the rats received a glucose load of 200 mgm. per 100 grams of rat per hour. Twelve pairs of adrenal enucleated rats were given 8 cc. of ACE per 24 hours. The addition of 2 parts per million of epinephrine did not improve work performance.

TABLE 2. THE WORK PERFORMANCE OF ADRENALLY INSUFFICIENT RATS GIVEN ADRENAL CORTEX EXTRACT AND GLUCOSE WITH AND WITHOUT EPINEPHRINE. GLUCOSE LOAD OF 200/100/h

ACE cc. 24 hrs.	Epinephrine concentration	Operation	Number of pairs	Total recorder revolutions. Averages and range	
				Epinephrine	Controls
8	1:500,000	enucleation	12	23177 (7091 –47556)	24843 (11028–42299)
12	1:500:000	enucleation	15	38702 (14521–53909)	39821 (26467–53753)
15	1:50,000	enucleation	6	20307 (6458–50342)	42002 (33493–53535)
15	1:500,000	adrenalectomy	19	45830 (30940–59031)	45946 (37760–60140)

Five of the 12 rats which received epinephrine died during 24 hours and 6 of the 12 rats without epinephrine died.

Fifteen pairs of adrenal enucleated rats received glucose with 12 cc. of ACE per 24 hours. The addition of 2 parts per million of epinephrine did not improve work performance. Two of the 15 rats which received epinephrine died during 24 hours and 1 of the 15 rats without epinephrine died.

Six pairs of adrenal enucleated rats received glucose with 15 cc. of ACE per 24 hours. The addition of 20 parts of epinephrine per million suppressed work. Four of the 6 rats which received epinephrine died during 24 hours and none of the rats without epinephrine died.

Nineteen pairs of surgically adrenalectomized rats received glucose with 15 cc. of ACE per 24 hours. The addition of 2 parts of epinephrine per million did not improve work. None of the rats died in this series.

TABLE 3. THE WORK PERFORMANCE OF ADRENALLY INSUFFICIENT RATS GIVEN ADRENAL CORTEX EXTRACT AND GLUCOSE WITH AND WITHOUT EPINEPHRINE. GLUCOSE LOAD OF 200/100/h. INFUSED THROUGH PLASTIC TUBE TIED INTO THE JUGULAR VEIN AND DIRECTED TOWARDS THE HEART

ACE cc. 24 hrs.	Epinephrine concentration	Operation	Number of pairs	Total recorder revolutions. Averages and range	
				Epinephrine	Controls
15	1:500,000	enucleation	9	42038 (15744–53960)	38584 (26162–49940)
15	1:100,000	enucleation	12	39949 (7793 –55458)	37380 (13520–55666)
15	1:500,000	adrenalectomy	20	43030 (25130–57325)	36927 (15111–52975)
Total averages with standard deviation of average				41910 (1731)	37688 (1484)
Difference in averages with standard deviation of the difference—4220 (2280)—Ratio—1.85					

In Experiment 3 (Table 3), the infusions were made through a plastic tube which was tied into the jugular vein and directed towards the heart. Nine pairs of adrenal enucleated rats were given glucose with 15 cc. of ACE per 24 hours. The animals which received 2 parts per million of epinephrine performed a greater average amount of work than did the animals without epinephrine. Similar results were obtained with 12 pairs of adrenal enucleated rats given glucose and 15 cc. of ACE per 24 hours when 10 parts per million of epinephrine were added and in 20 pairs of surgically adrenalectomized rats given glucose and 15 cc. of ACE per 24 hours when 2 parts per million of epinephrine were added. Three of the 41 rats given epinephrine in this experiment died during 24 hours and 6 of the rats without epinephrine died. It was noted that a number of the rats in this experiment exhibited respiratory distress and sharp decreases in work output associated with a thrombus in the vessels of the heart or lungs. These complications occurred more frequently among the animals which did not receive epinephrine. When the results for all of the animals which received epinephrine in Experiment 3 were grouped together, the average was 41,910 recorder revolutions with a standard devia-

tion of the average of 1,731. The average for the control animals was 37,688 recorder revolutions with a standard deviation of the average of 1,484. The ratio between the difference (4,220) and the standard deviation of the difference (2,280) was 1.85. From the statistical standpoint the probability is 93 chances in 100 that the performance of the rats treated with epinephrine in Experiment 3 was better than the performance of the controls. It is concluded, however, that the group difference was probably based upon the lower frequency of circulatory complications in the epinephrine-treated rats and therefore represents an artifact of methodology rather than any direct benefit of epinephrine upon the ability of the adrenally insufficient rat to sustain work output.

DISCUSSION

Although epinephrine may cause a temporary increase in the work output of the adrenally insufficient rat, just as it does in fatigued normal animals, it fails to increase the total energy output of the animal when given alone or in combination with ACE. Large doses of epinephrine caused a decrease in work. Adrenal demedullated rats show normal voluntary activity (Ingle and Harris, 1936) and a normal total work output when subjected to stimulation of the gastrocnemius muscle (Ingle, Hales and Haslerud, 1936) although it was observed (Ingle and Lukens, 1941) that the adrenal demedullated rat shows a lower than normal rate of work during the first few hours of muscle stimulation. It is not clear as to whether this difference was due to the absence of the adrenal medulla or to a decrease in the secretory activity of the regenerated cortex of the demedullated gland. The physiological role of the adrenal medulla has remained difficult to clarify because any means of inactivating this portion of the gland may possibly limit the secretory activity of the cortex. All of the data from this and previous studies by one of us (D.J.I.) are consistent with the concept that if the adrenal medulla plays a role in body economy, it is manifest principally in acute emergencies rather than by sustaining resistance to prolonged stress.

The data from surgically adrenalectomized animals were in agreement with the results from animals having the adrenal glands inactivated by enucleation without surgery. There was some evidence (Experiment 3, Table 3) that epinephrine reduced the number of complications resulting from the intravenous administration of hypertonic solutions of glucose. The group differences in work (Table 3), if reliable, would seem to represent an example of an artifact in results rather than a true beneficial effect of epinephrine upon work.

SUMMARY

Surgically adrenalectomized rats and adrenal enucleated rats were subjected to faradic stimulation of both hind legs five times per

second until death or for 24 hours. Each rat (200 grams body-weight) received continuous intravenous infusions at the rate of 20 cc. per 24 hours. The solution contained 0.9 per cent sodium chloride, 0.1 per cent ascorbic acid, various concentrations of adrenal cortex extract (ACE), and epinephrine was added to the solution given one animal of each pair. Glucose was added in some experiments. The total work output was proportional to the amount of ACE administered but the addition of epinephrine was without apparent direct effect upon total work. Epinephrine in amounts of 20 and 40 parts per million suppressed work. Work was greatly improved by the addition of 200 mgm. of glucose per 100 grams of rat per hour.

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A NEW REAGENT FOR THE HISTOCHEMICAL DEMONSTRATION OF ACTIVE CARBONYL GROUPS. A NEW METHOD FOR STAINING KETONIC STEROIDS[1]

RIVKA ASHBEL[2] AND ARNOLD M. SELIGMAN

From the Kirstein Laboratory for Surgical Research, Beth Israel Hospital, and the Department of Surgery, Harvard Medical School, Boston, Massachusetts

PRESENT methods for demonstrating the carbonyl groups of ketosteroid are based on their reaction with phenylhydrazine (Bennett, (1940) or with 2, 4-dinitrophenyl hydrazine (Dempsey and Wislocki, 1946; and Albert and Leblond, 1946) to form colored hydrazones (yellow or orange). Further evidence that these fat-soluble, carbonyl-containing substances were indeed ketosteroids was provided by localization of other steroid reactions and properties in the same areas as the positive phenylhydrazine reactions. These were fluorescence, birefringence, anisotropism after reaction with digitonin, Liebermann-Burchardt test, Schiff reaction, reduction of alkaline silver solutions, and sudanophilia (Dempsey, 1946). Still other evidence was afforded by correlation of the steroid reactions with different physiological states (Dempsey and Wislocki, 1946; Albert and Leblond, 1946; Greep and Deane, 1947; Deane and Shaw, 1947; Deane and Greep, 1947; and Wislocki, 1949).

By the application of these techniques, ketosteroid has been demonstrated in the outer portion of the zona fasciculata of the adrenal cortex in cats and dogs, and in the zona glomerulosa as well in rats and humans (Bennett, 1940; Dempsey and Wislocki, 1944; Deane and Greep, 1946; and Dempsey and Wislocki, 1946), in the interstitial cells of the mammalian testis (Pollack, 1942), in the cells of the theca interna of the follicle and the luteal cells of the rat's ovary (Dempsey and Bassett, 1943) and in the syncytial trophoblast of the human placenta (Wislocki and Bennett, 1943).

In order to improve the sensitivity of the phenylhydrazine reac-

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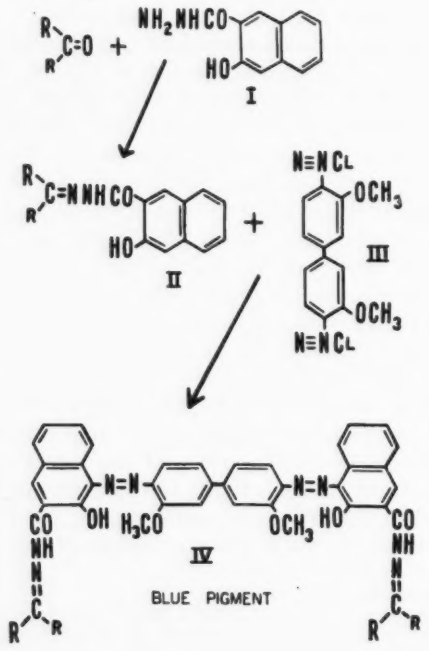
[2] On leave of absence from the Hebrew University, Jerusalem, Israel. Research fellow in surgery, Harvard Medical School. This fellowship was made possible by a grant from the Jane Coffin Childs Memorial Fund for Medical Research, Yale University.

tion by increasing the color value of the final compound, reagents were synthesized which contain a hydrazine group and which react with active carbonyl groups to form nearly colorless substances, but which may be converted by coupling with a tetrazonium compound into an intensely blue azo dye (Seligman and Ashbel, 1949). The best reagent for this purpose proved to be the hydrazide of 2-hydroxy-3-naphthoic acid (I) (Seligman, Friedman, and Herz, 1949). This was found to react *in vitro* with 3, 17, and 20-ketosteroids, but, as was expected, not to react with 11-ketosteroid. Frozen sections, prepared from formalin-fixed tissue, were washed free of formalin and reacted with the hydrazide (I) in alcohol-water-acetic acid solution. The reaction product (II) was insoluble in alcohol-water, so that the excess reagent was readily removed by washing. The frozen sections were then treated in 50 per cent alcohol at pH 7.2 with tetrazotized diorthoanisidine (III).[3] The development of a blue pigment (IV) in the areas which contained the lipoid with active carbonyl groups was rapid.



[3] This material is available commercially under the name of Dupont Naphthanil Diazo Blue B in stable, powder form and contains 20% tetrazotized diorothoanisidine, 5% zinc chloride, and 20% aluminum sulfate. It was provided through the courtesy of Dr. E. R. Laughlin, Dupont de Nemours and Co., Boston, Mass.

METHOD

Fresh tissue was fixed in 10 per cent formalin, neutralized by standing over calcium carbonate or with phosphate buffer (Lillie, 1948). The period of fixation was varied from several hours to several weeks, and in a few experiments for several months, without significant change in the final picture. In a few instances formalin was perfused through the tissue by intracardiac injection. After fixation the formalin was removed by washing the blocks in running tap water for 24 hours, or frozen sections (15–30 microns in thickness) were prepared and were washed in several changes of water for a few hours or longer.

The washed frozen sections were then placed at room temperature for 1–3 hours in an 0.1 per cent solution of 2-hydroxy-3-naphthoic acid hydrazide (I). This solution was prepared by dissolving the finely powdered hydrazide in 50 per cent ethyl alcohol (prepared from aldehyde-free absolute ethanol) containing 5 per cent glacial acetic acid, with the aid of warming (60–70°C.) and shaking. This solution could be stored at room temperature for periods as long as 12 days without alteration. The period of reaction with the tissue could be shortened to 30 minutes by incubation at 50°–60°C. The sections were then washed in several changes of 50 per cent alcohol for 2 hours followed by several changes of water for 2 hours.

The sections were then placed in a solution prepared from equal volumes of absolute alcohol and 1/15M phosphate or acetate buffer, pH 7.2–7.5. To 50 cc. of this solution containing the sections at room temperature, 50 mgm. of the stabilized tetrazonium powder (III) was added and stirred-in or a very fresh solution of the powder in a few cc. of cold water was added and stirred. The development of a blue color in the tissue reached a maximum of intensity in 0.5–2 minutes. The yellow solution also darkened during this period. The sections were transferred with a glass rod to water and washed with a few changes of water for 5–15 minutes. The sections were then mounted on glass slides with Kaiser's glycerogel. To avoid bubbles the sections were covered with glycerogel and were placed in the incubator for a few minutes before the cover slip was applied. The azo dye has not changed in the sections in over one year.

In general the shorter periods of incubation and of washing were used with the thinner sections. The periods of washing given above are considerably longer than was actually necessary by a safe margin. It was found that the addition of the tetrazonium compound to the sections in buffer-alcohol was a critical step in the procedure, because when the sections were added to the buffer-alcohol in which the tetrazonium compound had been dissolved previously, the development of color was poor. The concentration of alcohol was also important.[4] This may have been due in part to the rapid decomposition of the tetrazonium compound which occurs in aqueous solutions and to the necessity of coupling in tissue sections in a two-phase system; i.e.

[4] While the manuscript of this paper was being prepared, a note appeared in *Nature* (Camber, 1949) describing the use of 2-hydroxy-3-naphthoic acid hydrazide for staining ketosteroid in the adrenal cortex with diazotized amines. The procedure outlined is unlike the procedure herein described, and corresponds closely to the procedure (conducted in aqueous solution) which we first used in October, 1947, but with which we were not satisfied, because the degree of staining was variable and weak as compared to results obtained with the method eventually evolved.

the steroids and their reaction product (II) formed with the hydrazide is dissolved in the fat droplets, and the tetrazonium salt in aqueous solution is poorly and slowly diffusible into lipoid. At higher pH than that recommended, decomposition of the tetrazonium salt proceeds at a more rapid rate and results in a yellowish-brown cast to the sections. At lower temperatures the decomposition of the tetrazonium compound is slower, but the reaction rate is also slower. This slowing of the reaction rate is not due to a slower rate of coupling with the napththol, which proceeds rapidly in aqueous media even at 4°C., but must be due to a decrease in the solubility in lipoid of the steroid-hydrazide reaction product (II).

RESULTS WITH TISSUES WHICH CONTAIN KETOSTEROID

Adrenal Gland

The adrenal glands from 15 normal *rats* (killed with ether) were stained by the present method for carbonyl groups (figs. 7 and 8). Blue color was produced in the zona glomerulosa (11 were intense, 3 were weak and 1 did not stain). Blue color in the zona fasciculata was seen in 14 and 1 did not stain. The zona reticularis stained in 7, weakly in 1 and not at all in 8. The lipoid droplets of the cells were stained, and in addition the dye occurred in diffuse, granular-appearing deposits in the cytoplasm. The nuclei were not stained (fig. 8). The adrenal medulla stained a slight pink color in some of the glands. In no instance was any blue color seen in this location. The period of incubation with the hydrazide varied from 20 minutes to 7 hours. Most of the sections were incubated for 1–3 hours. No correlation of time and degree of staining was noted within these limits.

The adrenal glands from 13 ether-killed *rabbits* which had received urine for the Aschheim-Zondek test for pregnancy (most were negative) were stained by the present method for carbonyl groups.

The zona glomerulosa was intensely blue in 10 and weak in 3. The zona fasciculata was intensely blue in all 13. The zona reticularis was stained in 4, weakly in 3, and not at all in 6. In some sections the

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PLATE I

FIG. 1. Dog adrenal. The fasciculata contains most of the lipoidal carbonyl groups. The glomerulosa (left) is paler than noted in other species. The reticularis contains scattered cells which were stained, unlike most of the specimens examined. ×50.

FIG. 2. Rat testis. Interstitial cells which contain blue-stained fat droplets in their cytoplasm. The nuclei are unstained. ×1500.

FIG. 3. Rat cerebellum. The white matter is deeply stained and the molecular layer of the cortex is delicately stained. The granular layer of the cortex contains blue-stained extensions of nerve fibers from the white matter. The blue-stained material was not extractable with lipoid solvents. ×15.

FIG. 4. Dog adrenal. All layers of the cortex stain blue for lipoidal carbonyl groups. The capsule (top) and the medulla (bottom) are stained pink (not indicative of carbonyl groups). × 50.

FIG. 5. Dog ovary. Corpus luteum (upper right) is stained intensely blue for lipoidal carbonyl groups. Follicles contained a variable amount of delicate blue in the granulosal cells. ×50.

FIG. 6. Rat placenta (near term). The syncytial cells contain blue-stained fat droplets. ×900.

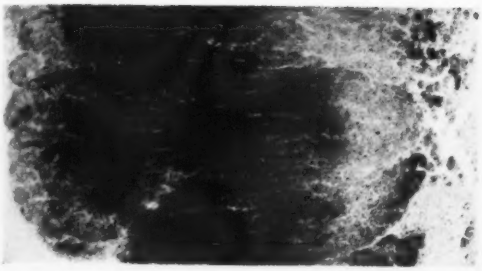


FIG. 1

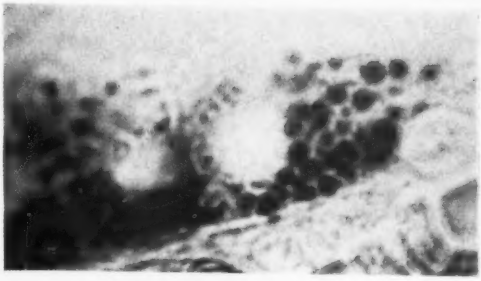


FIG. 2

FIG. 3

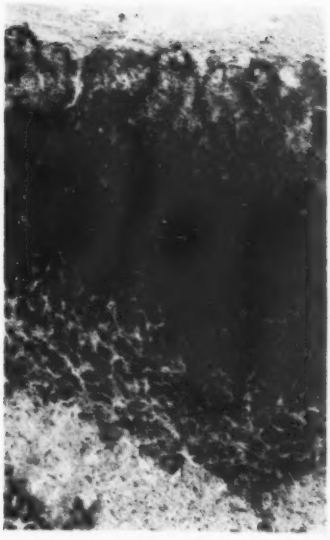


FIG. 4

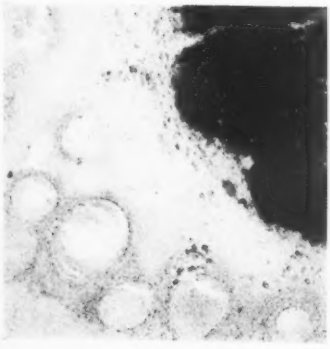


FIG. 5

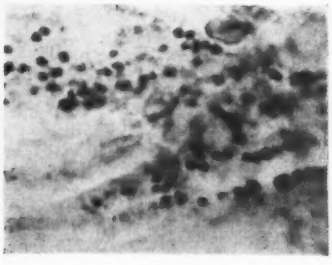


FIG. 6

reticularis was stained in one part of the section and not in another. The period of reaction with the hydrazide varied from ½–24 hours. However, most of the reactions were conducted for 1–3 hours. In some sections the adrenal medulla stained a faint pink and, rarely, a few cells were blue.

The adrenal glands from 12 normal *dogs* (morphinized and killed by exsanguination) were stained for carbonyl groups (figs. 1 and 4). The zona glomerulosa was blue in 8, very weak in 3, and not stained in 1. The zona fasciculata was intensely blue in 11 and not stained in 1. The zona reticularis was blue in 3 and not stained in 9. The medulla, fibrous capsule (fig. 4) and septa were pinker than in the other species described above (see page 573 for significance of pink color). The period of reaction varied from ½–18 hours; in most cases for 1 hour.

The adrenal glands obtained at post-mortem from 4 normal *humans*[5] (accidental deaths) were stained for carbonyl groups. All three layers of the adrenal cortex were stained blue. In 2 the glomerulosa and reticularis were more intensely stained than the fasciculata, in 1 all layers were equally stained, and in 1 the fasciculata was more intensely stained than the glomerulosa and reticularis. The medulla, fibrous capsule and septa were pinker than in the case of the dog. The period of incubation with hydrazide was 1–3 hours. Variation of this period in sections of the same gland did not change the results.

Sudanophilia was seen in all the layers of the cortex regularly. The carbonyl reaction not only varied in the different layers of the cortex, but occurred in fewer lipoid droplets than were stained with Sudan III. Mainly the smaller sudanophilic droplets appear to be involved in the carbonyl reaction.

Sections from the glands described above were used in experiments to test the blocking action of hydrazine hydrate, phenylhydrazine, hydroxylamine, 2-naphthoic acid hydrazide, and the effect of lipoid solvents such as acetone and alcohol. The blocking experiments with lipoid solvents were more efficient in preventing the blue reaction than were attempts to remove the blue compound with acetone extraction after the sections were stained. In both cases a delicate, lipoid-insoluble blue stain was noted in the lining membranes of the sinusoids of the adrenal cortex (fig. 9).

Occasional nerves cut longitudinally were stained a brilliant blue. This stain was not removed by acetone or alcohol extraction. Solvent treatment, therefore, enhanced the appearance of nervous tissue in these sections by eliminating the carbonyl reaction of lipoid.

Ovary

The ovaries of several gravid and non-gravid *rats* and *mice* were investigated. The cytoplasm of the lutein cells contained blue dif-

[5] This material was provided through the courtesy of the Mallory Institute, Boston City Hospital, Boston, Mass.

fusely dispersed dye. The nuclei were unstained. Cells of the theca interna in the outer portions of the corpora lutea were more intensely stained than the lutein cells in the center, as were also special stellate cells scattered throughout the corpus luteum, described for human ovaries by White, Hertig, Rock, and Adams, 1949. Cells of the interstitial glands of the ovary contained blue fat droplets. Theca folliculi also was stained blue. In addition the lining cells of the sinusoids of the corpora lutea were stained blue. The capillary stain in contrast to other areas was not removed by lipoid solvents, nor was it prevented by preparing paraffin embedded sections. The inner elastic layers of some blood vessels were stained blue.

The ovaries of 2 normal *dogs* were examined by this method. The corpora lutea were stained intensely blue (fig. 5). The cytoplasm of the lutein cells contained many blue fat droplets of various sizes. Young follicles exhibited a delicate violet pattern in the cytoplasm of the ova (fig. 12), and the theca folliculi cells contained bluish-violet staining nuclei (fig. 14). The stain in these cells was not altered by extraction with lipoid solvents. The older follicles were darker blue, due presumably to staining of the granulosal cells (fig. 13). The internal elastic layer of blood vessels of the ovary were stained blue.

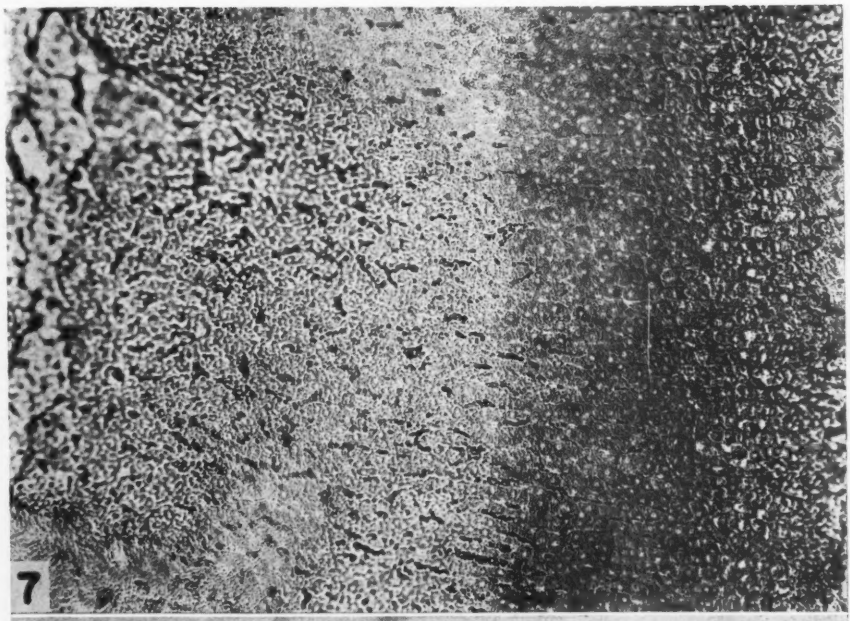
One dog was 6 weeks post-partum. The large corpora lutea were intensely blue. The dense fibrous septa were violet-pink, and the fine reticulum of the intercellular boundaries were blue. The lutein cells were large and vacuolated (figs. 16 and 17). They contained blue lipoid droplets and blue finely dispersed dye. This was especially noticeable in the theca cells of the periphery of the corpus luteum. In some sections the theca folliculi contained cells whose nuclei stained purple. They stain was not removed by lipoid solvents. These cells were not easily distinguished from the remaining ovarian stroma in eosin-hematoxylin sections.

Corpora lutea of *humans* stained less intensely than in the dog. The stain was finely dispersed in the cytoplasm of the thecal lutein cells (fig. 15, 24 days after menstruation). The nuclei were not colored. Darkly stained stellate cells, similar to those described by White *et al.* were scattered throughout the corpus luteum in a radiating pattern. Cells of the theca externa also stained blue, but this blue dye was not readily removed by lipoid solvents. The interstitial cells of a corpus atreticum were stained blue, but most of the blue colored material was not lipoid soluble.

PLATE II

FIG. 7. Rat adrenal. The reaction for lipoidal carbonyl groups is evident in the glomerulosa (right) and part of the fasciculata. Sinusoids in the fasciculata, reticularis and medulla were also stained, but this colored material was not extractable with lipoid solvents. Photographed through a red filter. × 180.

FIG. 8. Same section as Fig. 7, showing more stain in the glomerulosa (right) than the fasciculata. The nuclei are outlined by the stained fat droplets and finely dispersed dye in the cytoplasm. Photographed through a red filter. ×1200.



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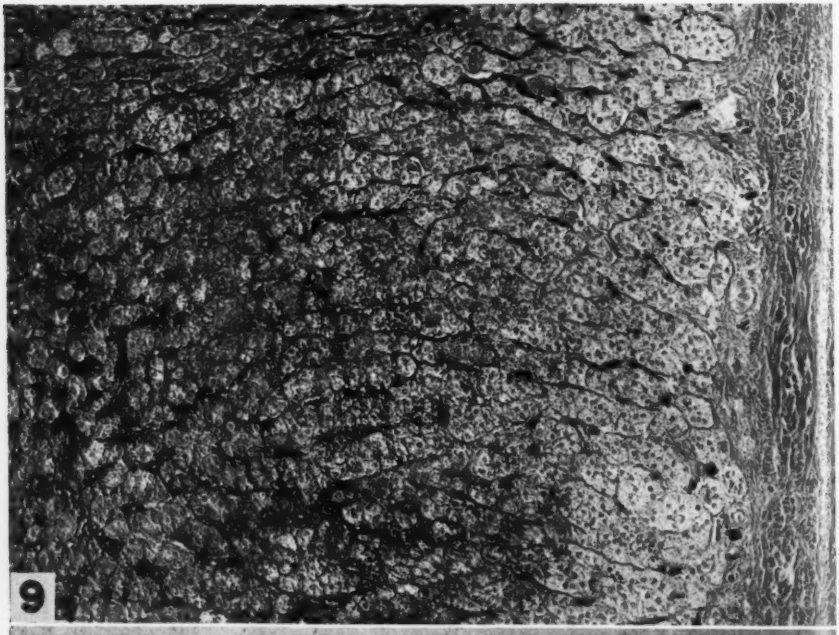
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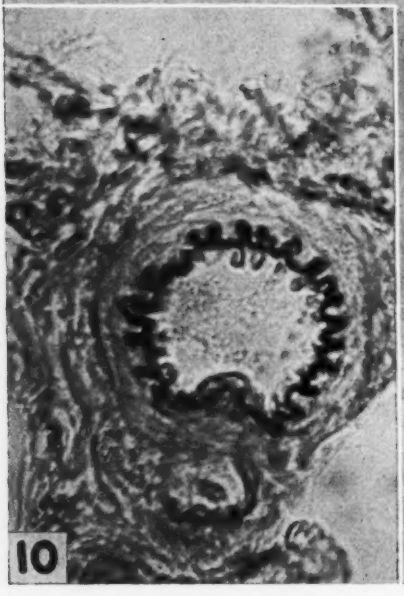
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11

Placenta

The placenta of a *rat* near term gave an intense blue color in lipoid droplets in the syncytial throphoblast of the placental labyrinth (figs. 6 and 18). In some areas the cytoplasm was stained diffusely blue, although not sufficiently to mask the droplets. The droplets varied in size and were located predominantly on the fetal side of the syncytium (fig. 18). In addition there was a positive reaction of the cytoplasm of the trophoblastic giant cells located around the margin of the placental disk.

The placentas of a *rat* and 6 *mice* from mid-pregnancy stained similarly but weakly. Of three *human* placentas at term, only one showed isolated, scattered, blue-staining droplets in the flattened syncytial trophoblasts.

Testis

The testis of 1 *rat*, 1 *rabbit*, and 3 *dogs* were stained for lipoidal carbonyl groups. Fat droplets in the cytoplasm of the interstitial cells stained blue. The nucleus was unstained and was outlined by the surrounding blue droplets (fig. 2).

Other tissues

Lipoidal carbonyl groups were not demonstrable by this method in lung, liver, kidney, spleen, pancreas, skeletal muscle, subcutaneous or mesenteric fat, intestine, uterus, prostate, epididymis, seminal vesicles, thyroid, pituitary, or blood smears.

REACTION WITH NON-LIPOID MATERIAL IN TISSUES

Fibrous tissues.

In some of the sections, particularly of human adrenal tissue, a pink color was noted in fibrous tissue (fig. 4). This could not be prevented by prolonged washing. It was probably due to attachment of traces of the hydrazide (I) to protein or collagen through the naphtholic group. Coupling with the tetrazonium compound under these circumstances would occur into the free hydrazino group of the hydrazide. In *in vitro* experiments, it was found that the tetrazonium compound gave a red color with the hydrazide (I). However, when the hydrazino-group was tied-up by reaction with acetone or for-

PLATE III

FIG. 9. Human adrenal cortex. Formalin-fixed tissue was carried through the paraffin embedding procedure, which removed lipoidal carbonyl-containing material. Only intercellular reticulum and sinusoids were stained blue. Photographed through a red filter. ×180.

FIG. 10. Dog arteriole in neurovascular bundle. Alcohol fixed. The inner elastic membrane is stained intensely blue. ×1200.

FIG. 11. Dog sciatic nerve. Single nerve fiber showing chicken-wire pattern in the myelin sheath. The blue colored material in this network was insoluble in lipoid solvents. ×2200.

maldehyde, coupling could only occur ortho to the hydroxy group. This type of coupling resulted in the production of a blue color. Treatment of stained sections with lipoid solvents such as absolute alcohol or acetone which slowly removed the blue compound, did not affect the non-specific pink color seen in fibrous tissue. This was considered evidence of attachment of the hydrazide (I) to protein in areas stained pink, and of attachment to lipoid carbonyl groups in areas stained blue. Further evidence was provided that the naphtholic group was important in attachment to tissue for the development of the pink color, by the preparation of 2-naphthoic acid hydrazide (Seligman, Friedman and Herz, 1949). When this material was used instead of the hydrazide (I), the sections were complete blanks (neither blue for ketosteroid nor pink in fibrous tissue). However, after blocking the carbonyl groups with this reagent, subsequent treatment with the hydrazide (I) led to development of the pink color in fibrous tissue, even though the blue color of carbonyl groups was permanently blocked. Other blocking agents such as hydrazine hydrate and hydroxylamine permanently blocked both the pink and blue colors after subsequent treatment with the hydrazide (I). This was probably due to the strong basic character of these agents.

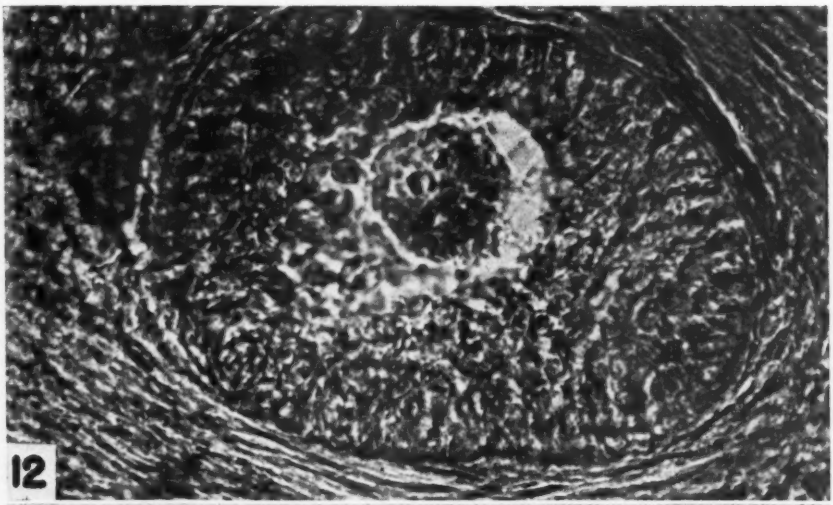
Nervous tissue

In some sections of adrenal gland which included segments of nerve, a brilliant blue color was produced by the reaction in the myelin sheaths. This pigment, however, was not removed by the lipoid solvents which successfully removed the blue stain produced by carbonyl groups in lipoid. Subsequent investigation revealed that this reaction occurred regularly in formalin-fixed white matter of central (fig. 3) and peripheral nervous tissue (fig. 11). The active carbonyl groups involved in the reaction with nervous tissue were found only after formalin fixation, or in alcohol-fixed tissue subsequently exposed to formalin. A weak reaction was also observed with alcohol-fixed tissue subsequently treated with mineral acid or mercuric chloride. Unlike the reaction with lipoid, the reaction persisted in formalin-fixed tissue which was carried through the paraffin embedding process. In all cases, the reaction was blocked by prior treatment with hydrazine hydrate. Fractionation of fresh white matter of brain revealed that the reaction occurred in the protein fraction only after subsequent exposure to formalin. This explains the discrepancy between the amount of aldehyde demonstrated in brain by the Schiff reagent and the small amount of aldehyde actually isolated

PLATE IV

FIG. 12. Dog ovary. A young follicle was stained a delicate bluish-violet in the cytoplasm of the ovum. The granulosa cells were not stained blue. ×800.

FIG. 13. Dog ovary. An older follicle stained intensely blue, presumably in the enveloping granulosa cells. ×800.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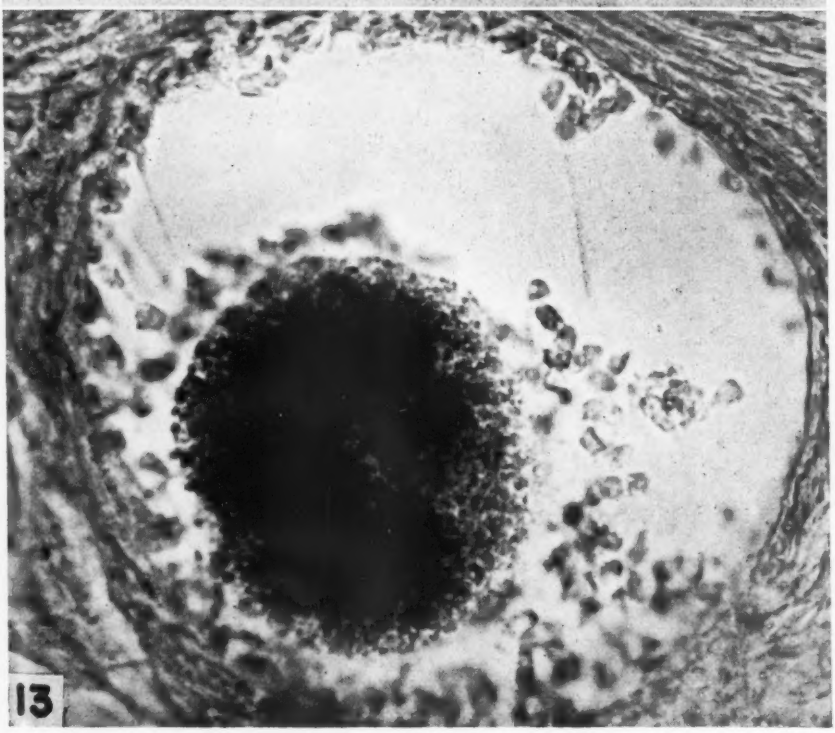
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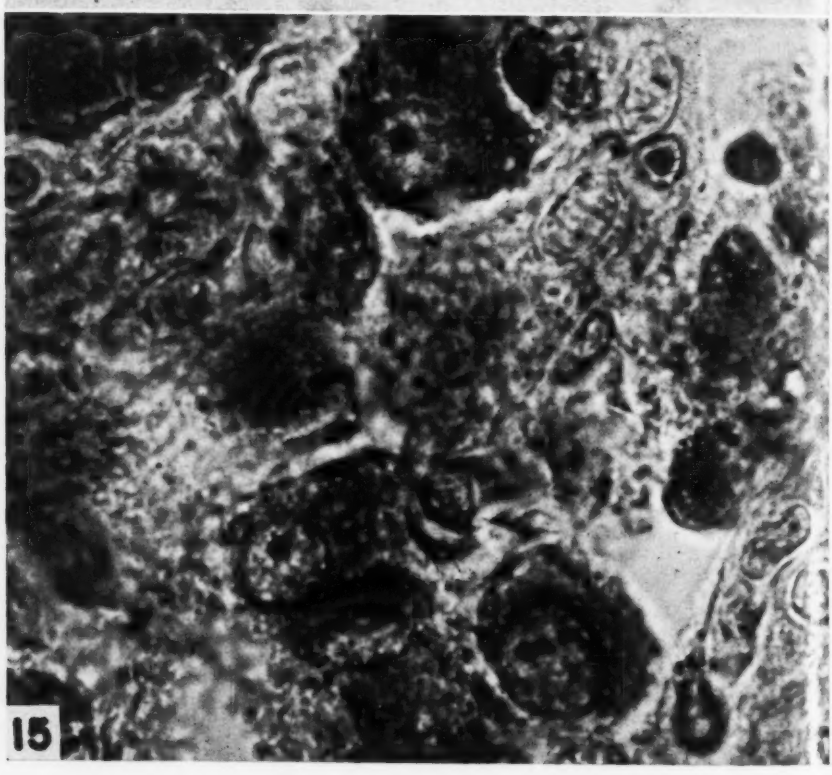
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from brain (Anchel and Waelsch, 1942). Further study of the results with nervous tissue will form the subject of another communication (Seligman and Ashbel, 1949).

Elastic tissue

The wavy elastic layer of large blood vessels was found to react with the hydrazide to give an intense blue stain (fig. 10). This stain was not removed by lipoid solvents and its production was blocked by prior treatment with hydrazine hydrate. However, the reaction differed from that of the adrenal cortex and nervous tissue, in that exposure to formalin was not required to unmask the carbonyl groups. This was demonstrated by successful staining of alcohol-fixed tissue. Similar staining of elastic tissue has been observed with other methods for demonstrating carbonyl groups (Feulgen and Voit, 1924; Albert and Leblond, 1946; Dempsey, 1946; and Shreck and McNamara, 1947).

Sinusoidal reticulum

After extraction with acetone of all stained lipoid in adrenal cortex, blue color remained in the lining membranes of many of the sinusoids (fig. 7). This was also observed in formalin-fixed tissue which had been carried through the paraffin embedding process (fig. 9).

IN VITRO REACTIONS WITH PURE MONOKETOSTEROIDS

In order to compare the relative reactivity of the 3, 11, 17 and 20 keto groups, which occur singly or in combination in various ketosteroids, compounds[6] were selected which contained only one of these keto groups in each. Thus, a 3-keto-group was tested in testosterone, an 11-keto-group in the methyl ester of 3-hydroxy-11-keto aetio cholonic acid, a 17-keto group in oestrone, and a 20-keto group in pregnen-β-3-ol-one-20. Estradiol and recrystallized cholesterol served as non-ketonic steroids.

Each steroid (5 mg.) was dissolved in 10 cc. of hot absolute ethanol and the solution was cooled to room temperature. A solution of the hydrazide (I) (10 mg.) in absolute ethanol (10 cc.) or in 50% alcohol

[6] These compounds in crystalline form were provided us through the courtesy of Professor Louis F. Fieser, Harvard University.

PLATE V

FIG. 14. Dog ovary (6 weeks post-partum). Theca folliculi cells stained purple in the nuclei. This colored material was not lipoid soluble. Photographed through a red filter. ×150.

FIG. 15. Human corpus luteum (24 days post-menstrual). Theca interna cells in the periphery of the corpus luteum contain fine, blue-stained, lipoid droplets in the cytoplasm. Nuclei were not stained. The nucleoli were not colored blue. Photographed through a red filter. ×2000.

was added, followed by 1 drop of glacial acetic acid. In some experiments the solution was heated to boiling and then cooled; in others, the reaction proceeded at room temperature for various periods. The reaction was terminated by the addition of several drops of freshly distilled pyruvic acid, which converted the excess hydrazide to an alkali soluble hydrazone. After standing for an hour or after heating to boiling, 25 cc. of benzene and 25 cc. of ethyl acetate was added and the mixture was exhaustively extracted with dilute sodium bicarbonate solution (5%) until the washings failed to color on addition of

TABLE 1. EXTENT OF REACTION OF 2-HYDROXY-3-NAPHTHOIC ACID HYDRAZIDE WITH PURE STEROIDS UNDER VARIOUS CONDITIONS

Compound	Position of the keto-group	Reaction in boiling benzene	Reaction in boiling alcohol for 1 minute and room temperature for 24 hours	Reaction in boiling alcohol for 1 minute	Reaction in alcohol at room temperature for one hour
Testosterone	3	+++++	++++	+++	0
3-Hydroxy-11-keto aeteo cholanic acid methyl ester	11	0	0	0	0
Estrone	17	+++	+++++	+++	+++
Pregnen-β-3-ol-one-20	20	+	+++	++	0
Estradiol	—	0	0	0	0
Cholesterol	—	0	0	0	0

the tetrazonium compound (III). The organic layer was then shaken with a solution of the tetrazonium compound containing a little sodium bicarbonate. The intensity of the blue-red dye in the organic layer indicated the extent of reaction of the hydrazide with the steroid. Table 1 gives the results of the reaction under various conditions. The most active carbonyl groups were 3 and 17. The 20-keto group was less reactive, and the 11-keto group did not react at all. The Schiff reagent did not react with any of these ketosteroids dissolved in dilute alcohol or dilute propylene glycol within a period of several hours, or with a solution of testosterone in tricaprylin (10 mg. per cc.).

SPECIFICITY OF THE REACTION

Doubt has been cast upon the specificity of the phenylhydrazine reactions for demonstrating ketosteroid in the lipoid of the various sites enumerated above by several authors (Gomori, 1942; Albert and Leblond, 1946; Oster and Oster, 1946; and others quoted in Dempsey, 1948), on the grounds that lipoidal aldehydes (stearyl and palmityl aldehydes) are readily liberated from plasmalogens by hydrolysis

PLATE VI

FIG. 16. Dog ovary (6 weeks post-partum). The lutein cells are vacuolated and contain a fine network of blue-stained fat droplets and granules at the cell periphery. Lipoid solvent extraction removed the blue material. Photographed through a red filter. ×800.

FIG. 17. Same section as Fig. 16. Eosin-hematoxylin stain. Cell nuclei and vacuolization are shown. ×800.

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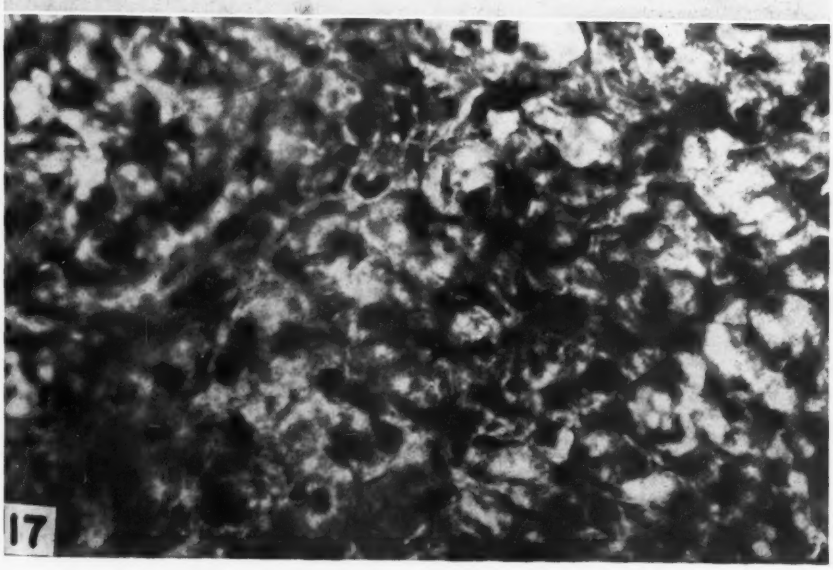
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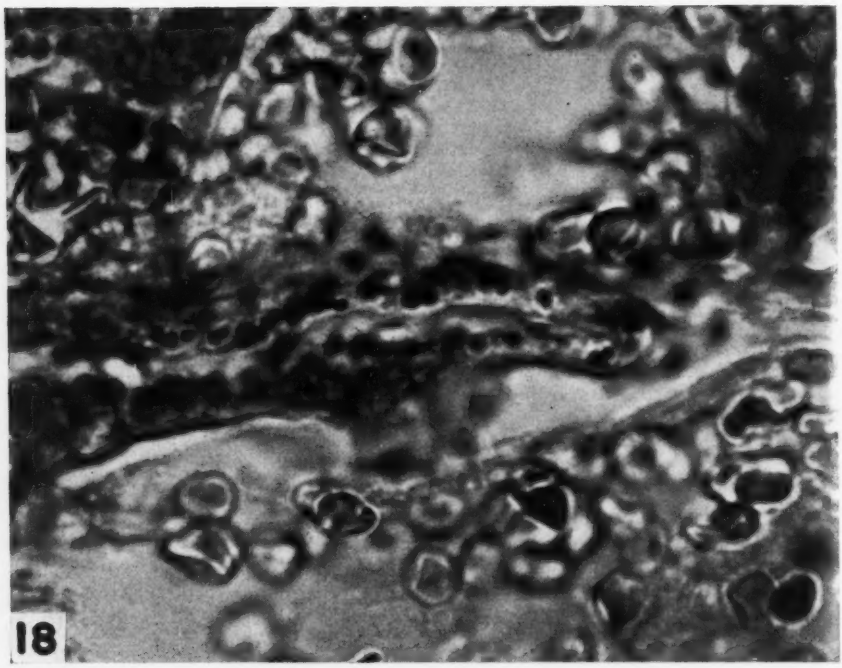

PLATE VII

FIG. 18. Rat placenta (near term). A chorionic villus surrounded by maternal blood. The syncytial cells contain blue fat droplets located on the fetal side of the cells. Photographed through a red filter. ×2000.

and are deomonstrated by the Schiff reagent (Feulgen and Voit, 1924). Plasmalogens have been demonstrated by this series of reactions in these glands and other tissues (Feulgen and Bersin, 1939). The structure of the plasmalogens was established by isolation of the aldehydes from muscle (Anchel and Waelsch, 1942). They are phospholipids in which the aldehydes are bound to glycerol through an acetal linkage and which, in addition, carry a phosphate ester of ethanolamine (Tannhauser and Schmidt, (1946). Since aldehydes react readily with hydrazines, the specificity of the methods for ketosteroid depends upon whether hydrolysis of plasmalogens occurs under the conditions given. The demonstration of plasmalogens in tissue by the Schiff reagent requires prior treatment with mineral acid or mercuric chloride, unless the Schiff reagent is itself sufficiently acid to produce hydrolysis. It is claimed that exposure of tissue to formalin for more than six hours will also hydrolyze the acetal and that further exposure to formalin will destroy the plasmal that has been liberated (Hayes, 1949). In the case of ketosteroid, formalinization of tissue is

necessary to unmask the carbonyl group. Once this is accomplished (one or more hours) deterioration of the staining reaction with further exposure to formalin (weeks) does not occur. This is in contrast to what is claimed for plasmal (Hayes, 1949).

Although the phenylhydrazine reaction occurs with both aldehydes and ketones, the Schiff reagent reacts readily with aldehydes, less readily with certain cyclic ketones (Karrer, 1938), presumably with easily oxidizable α-ketols, and not at all with most ketones. The Schiff reagent has been reported to react *in vitro* with pure steroids (Dempsey and Wislocki, 1944; Dempsey, 1946). However, others claim that ketosteroids failed to react with the Schiff reagent (Albert and Leblond, 1946; and Oster and Oster, 1946). The four monoketosteroids used above for studying the hydrazine reaction failed to react with the Schiff reagent. This would suggest that the Schiff reagent demonstrates aldehyde or possibly α-ketol, and that the hydrazine reaction can demonstrate aldehyde and ketosteroid. It is claimed that the Schiff reaction occurs in exactly the same locations as the hydrazine reaction (Albert and Leblond, 1946). In pilot experiments with adrenal gland of rabbit and ovary of dog, in which the period of fixation in neutral formalin was varied from 15 minutes to weeks, it was found that the blue stain developed with the hydrazide reaction was maximal after 1 hour's fixation and did not change significantly in intensity one way or another after longer fixation; nor did the location of the reaction change. No reaction with the Schiff reagent occurred with adjacent sections fixed for a short time or for very long periods. Other sections treated with mercuric chloride for 5–10 minutes gave the same degree of staining or slightly more intense staining with the hydrazide, and in some sections of unfixed adrenal gland so treated gave a blue color only in the reticularis and medulla where ketosteroid is rarely found. Mercuric chloride did not unmask ketosteroid in the way that formalin fixation did. Sections fixed in formalin for 4 hours and then stained were less blue than similar sections treated with mercuric chloride. In the latter, reticularis and medulla were also blue. Furthermore, plasmal has been isolated from skeletal muscle (Anchel and Waelsch, 1942) but the carbonyl reaction with the hydrazide gave consistently negative results with formalin-fixed muscle.

Proof of the specificity of the hydrazine reaction for ketosteroid, when conducted as herein described, hinges upon whether the carbonyl groups are aldehydic or ketonic. Reactions which depend upon the relative reactivity of aldehydes and ketones with reagents such as Schiff, cannot settle the question definitely. Conclusive proof would be afforded by a reaction which depended upon whether the carbonyl group carried a hydrogen atom (HC—R) or only carbon (R—C—R),

$$\underset{\underset{\|}{O}}{HC}\text{—R} \qquad \text{R—}\underset{\underset{\|}{O}}{C}\text{—R}$$

rather than upon the reactivity of the carbonyl group itself. Such a reaction is available. The phenylhydrazones of aldehydes couple with diazonium compounds in pyridine (Seligman, Gofstein, and Rutenburg, 1949) to form colored water-insoluble formazans. The phenylhydrazones of ketones cannot undergo the reaction because of the absence of replaceable hydrogen. Utilizing this reaction and tetrazotized diorthoanisidine, a blue (benzaldehyde, furfural) or dark red (formaldehyde, butyraldehyde) diformazan is formed from the appropriate phenylhydrazone. This new histochemical reaction for aldehydic carbonyl groups forms the subject of another communication (Seligman and Ashbel). Preliminary experiments with this method suggest that free aldehydes are not present in adrenal cortex fixed in neutral formalin for 24 hours and not exposed to strong hydrolytic agents.

It may, therefore, be concluded that the hydrazide reaction with carbonyl groups of lipoid demonstrates ketosteroid in tissue, after prior fixation in formalin from 1 hour to many weeks.

SUMMARY

A new method for demonstrating carbonyl groups in the lipoid of formalin-fixed tissues is described. The method is based on the reaction of aldehydic and ketonic groups with 2-hydroxy 3-naphthoic acid hydrazide followed by coupling of tetrazotized diorthoanisidine into the naphtholic ring with the production of a blue, insoluble azo compound. Reaction with certain carbonyl groups of non-lipoid material in nervous tissue, elastic tissue, and reticulum is described. Carbonyl-reacting lipoid was found in adrenal cortex, corpus luteum, interstitial cells of testis and syncytium of placenta. The localization in tissue was similar to that previously demonstrated with other methods. Evidence is presented that the carbonyl-containing lipoid in these formalin-fixed glands is in fact ketosteroid.

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A NEW REAGENT FOR THE HISTOCHEMICAL DEMONSTRATION OF ACTIVE CARBONYL GROUPS. THE PREPARATION OF 2-HYDROXYNAPHTHALENE CARBOXYLIC AND SULFONIC ACID HYDRAZIDES[1]

ARNOLD M. SELIGMAN, ORRIE M. FRIEDMAN, AND JOSEF E. HERZ

From the Kirstein Laboratory for Surgical Research, Beth Israel Hospital, the Department of Surgery, Harvard Medical School, and the Chemical Laboratories of Harvard University

BOSTON, MASSACHUSETTS

In order to develop a histochemical method for active carbonyl groups, which would result in the production of intensely colored, insoluble dyes, acid hydrazides of several naphthols were prepared. It was expected that coupling of a suitable diazonium compound into the naphthol, after reaction of the hydrazide group with carbonyl, would yield azo dyes of suitable intensity for microscopic delineation.

THE hydrazides of three hydroxy naphthoic acids and 2-hydroxy-6 naphthalene sulfonic acid were prepared. The sulfone hydrazide failed to react with ketosteroid in adrenal cortex under conditions in which the carboxylic acid hydrazides did react (Ashbel and Seligman, 1949). The pigment produced on coupling with diazotized diorthoanisidine, after reaction of the three naphthoic acid hydrazides with ketonic material, was blue (2-hydroxy-3-naphthoic acid hydrazide), brownish purple (1-hydroxy-2-naphthoic acid hydrazide), and violet (2-hydroxy-6-naphthoic acid hydrazide). The first pigment was most suitable for histochemical study and the hydrazide was readily prepared. The preparation of the four hydroxy hydrazides is described below. In addition the preparation of 2-naphthoic acid hydrazide is given. This material was used for control studies (Ashbel and Seligman, 1949).

EXPERIMENTAL[2]

2-Hydroxy-3-naphthoic acid hydrazide

The acid chloride was prepared from 20 gms. of 2-hydroxy-3-naphthoic acid (Eastman technical grade) by warming with 25 cc. of thionyl chloride until all the solid had dissolved and hydrogen chloride

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[1] This investigation was aided by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Microanalyses by Shirley Katz. All melting points are corrected.

was no longer evolved (Meyer, 1901). The excess thionyl chloride was removed by distillation at reduced pressure. The acid chloride solidified on cooling and dissolved with the evolution of heat upon addition of 50 cc. of absolute methyl alcohol. The alcohol was removed by distillation at reduced pressure. To the low melting ester that was formed, was added 35 cc. of 85 per cent hydrazine hydrate and the mixture was heated on the steam bath for 3 hours (Franzen and Eichler, 1908). The reaction period was shortened by use of 100 per cent hydrazine hydrate. The hydrazide separated from the reaction mixture as a crystalline mass on cooling. Water was added, the crystals were collected with the aid of suction and washed with water. The crystals were dissolved in 500 cc. hot alcohol. After treatment with active charcoal, the solution was chilled and the crystals collected and washed with ether. The pale-yellow platelets melted at 203–4°. The yield was 16 gms. (75 per cent). The reported melting point is 203–4° (Franzen and Eichler, 1908).

1-Hydroxy-2-naphthoic acid hydrazide

This hydrazide was prepared from 1-hydroxy-2-naphthoic acid (Eastman), exactly as described for the 2, 3 isomer above. It was obtained as fine, pale-tan needles, melting at 212–213°. The same melting point is given by Beyer and Schulte, 1941.

2-Hydroxy-6-naphthoic acid

Attempts to prepare 2-hydroxy-6-naphthoic acid according to the method of Howarth and Sheldrick (1934) gave poor results. This material was prepared from the nitrile by hydrolysis with hydrobromic acid in acetic acid. 2-Hydroxy-6-bromonaphthalene was prepared from beta naphthol (Organic Syntheses, 1940) and methylated with methanol and sulfuric acid (Woodward and Eastman, 1944). A mixture of 90 gms. of 2-methoxy-6-bromonaphthalene and 60 gms. of copper cyanide was heated for one hour at 240°C. The mass dissolved and became brown. The liquid was poured onto a cold surface and pulverized. The powder was warmed in a solution of 300 cc. of concentrated ammonia in 1 liter of water. The mixture was filtered and the residue was distilled at reduced pressure. The nitrile was obtained in 65–70 percent yield, b.p. 205–8°, 14 mm.; m.p. 103°. This material (14 gm.) was heated under reflux overnight with 30 cc. of glacial acetic acid and 100 cc. of 48 per cent hydrobromic acid. Upon cooling, the hydroxy acid crystallized. It was dissolved in ethanol, treated with norite, and after the addition of water, crystallized as tan needles melting at 241°; yield 11 gms. (78 per cent).

2-Hyrdoxy-6-naphthoic acid hydrazide

The ethyl ester was prepared by solution of 1.5 gms. of 2-hydroxy-6-naphthoic acid in 15 cc. of absolute ethanol and by saturation of

the solution with dry hydrogen chloride. After standing for 12 hours the mixture was diluted with water. The oil that separated crystallized slowly on standing. The crude ester was warmed on the steam bath with 2 cc. of 85 per cent hydrazine hydrate for 1 hour, at which time the hydrazide had separated as a white crystalline solid. After recrystallization from alcohol, the product melted at 256–8°; 0.5 gm. A second crop 0.3 gm. (m.p. 254–6°) was obtained by concentration of the mother liquor.

Analysis calculated for $C_{11}H_{10}N_2O_2$: C, 65.35; H, 4.96
Found: C, 65.33; H, 4.96

2-Hydroxy-6-naphthalene sulfonhydrazide

The required 2-carbethoxy-6-naphthalene sulfonic acid was prepared (Zincke and Dereser, 1918) by the slow addition of 21 cc. of ethyl chlorocarbonate to a vigorously stirred solution of 49.2 gms. of sodium 2-hydroxy-6-naphthalene sulfonate (Eastman technical grade) and 8 gms. sodium hydroxide in 200 cc. of water. The stirring was continued for an additional hour, after which the mixture was cooled to precipitate the carbethoxy derivative. This material was collected with the aid of suction and dried by warming on the steam bath. The crude product (44 gms.) was ground with an equal weight of phosphorus pentachloride and was heated on the steam bath for 2 hours (Zincke and Dereser, 1918). The excess phosphorus pentachloride was decomposed by grinding the fused melt with ice. The acid chloride was filtered and after recrystallization from acetic acid melted at 116–18°, 15 gms.

The carbethoxy acid chloride (1 gm.) was heated for an hour on the steam bath with 5 cc. of 85 per cent hydrazine hydrate in which it dissolved. The solution was evaporated to dryness under reduced pressure and the residue was extracted with boiling methanol. After concentration and cooling, fine white needles were obtained. After two crystallizations from methanol the melting point without decomposition was 188°; yield 0.5 gm. (65 per cent).

Analysis calculated for $C_{10}H_{10}O_3N_2S$: C, 50.41; H, 4.19
Found: C, 50.46; H, 4.09

2-Naphthoic acid hydrazide

Beta naphthoic acid (10 gms.) was converted to the acid chloride, the methyl ester, and finally the hydrazide exactly as described for 2-hydroxy-3-naphthoic acid hydrazide. The product crystallized from alcohol-water in white needles melting at 146–8°; yield 6.8 gms. (62 per cent). The reported melting point is 147.5° (Goldstein and Cornamusaz, 1932).

SUMMARY

The preparation of five acid hydrazides is given. 2-Hydroxy-3-naphthoic acid hydrazide was found to be the most convenient to

prepare, and to be the most suitable for the histochemical reaction with steroidal carbonyl groups.

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NOTES AND COMMENTS

1-METHYL-2-MERCAPTOIMIDAZOLE: AN ANTITHYROID COMPOUND HIGHLY ACTIVE IN MAN[1]

THE antithyroid compounds used for the treatment of hyperthyroidism were previously selected for clinical trial on the basis of high potency in the rat (Astwood, Bissell and Hughes, 1945). Subsequent experience, showing that the rat assay was a poor index of effectiveness in man, led to the de-

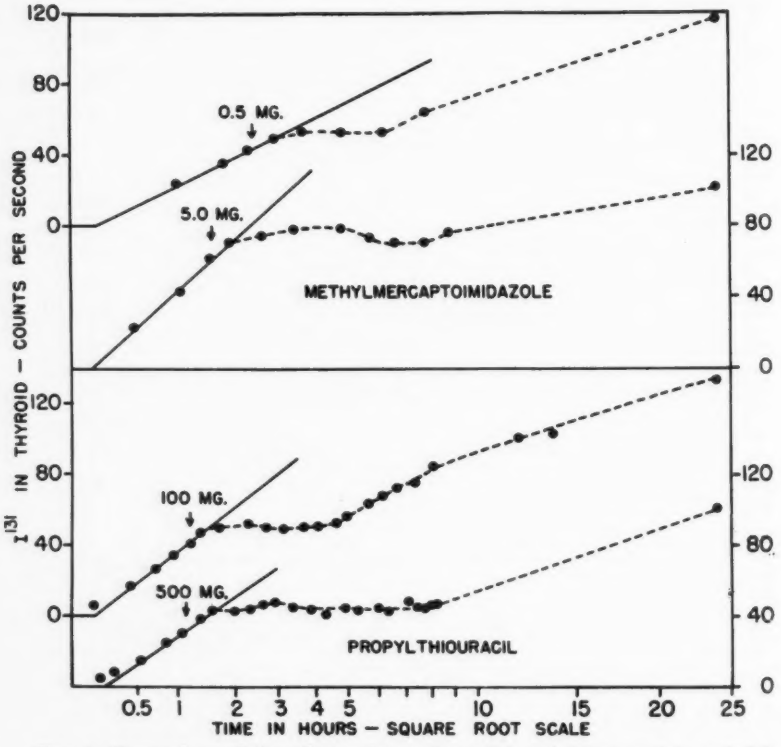

FIG. 1. Comparison of the effects of two doses, 0.5 and 5.0 mg. of 1-methyl-2-mercaptoimidazole with two doses, 100 and 500 mg., of propylthiouracil on the course of the iodine uptake by the thyroid gland in normal human subjects. Five-tenths mg. of 1-methyl-2-mercaptoimidazole was somewhat less effective than 100 mg. of propylthiouracil, while 5 mg. of the former exerted a more sustained effect than 500 mg. of the latter. The ordinate is an arbitrary scale of counts per second and the abscissa is the square root of the elapsed time after the oral administration of 100 microcuries of I^{131}. On this plot, a deviation from the normal straight line course of accumulation during the first 8 or 10 hours indicates inhibition.

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[1] This work was supported in part by grants from the Committee on Endocrinology of the National Research Council and from the American Cyanamid Company.

TABLE 1. EFFECT OF VARIOUS DOSES OF ANTITHYROID COMPOUNDS ON THE I^{131} UPTAKE BY THE THYROIDS OF NORMAL HUMAN BEINGS

Compound	Degrees of response*									Relative activities (thiouracil = 1.00)	
	Dose in milligrams										
	0.5	1	2.5	5	10	25	50	100	500	Man	Rat†
2-Thiouracil						1	2, 3	3, 4, 4		1.0‡	1.0
6-Methylthiouracil						2	3, 4	4		2.0‡	1.0
6-*n*-Propylthiouracil							1, 1, 2	3, 3, 3 3, 3	4	0.75‡	11.0
2-Mercaptoimidazole				0, 1, 2 2, 2, 3, 3	4, 4 2, 3, 4	4, 5		5		10.0‡	2.0
1-Methyl-2-mercapto-imidazole	3, 3, 3	2, 4	4	4, 5	4, 5	5, 5				100.0	2.0

* The degrees of response were classified as follows: 0 = no effect, 1 = slight or questionable effect, 2 = distinct but incomplete inhibition, 3 = complete inhibition for less than 4 hours, 4 = complete inhibition for more than 4 but less than 24 hours, 5 = complete inhibition for 24 hours or longer.
† Reference 2 and unpublished data.
‡ Reference 1.

velopment of a method using I^{131} by which these substances were tested directly in normal human beings (Stanley and Astwood, 1947).

Antithyroid activity determined in this way correlated quite well with clinical effectiveness. 2-Mercaptoimidazole, which was found to be about ten times as active as thiouracil, has now been used to treat thirty-four patients during a period of two years. The results obtained were quite in keeping with the predictions of its potency based on the iodine uptake test.

During the course of testing a limited number of further compounds by the radioiodine method, it was observed that 1-methyl-2-mercaptoimidazole was remarkably active. As little as 0.5 Mg. exerted a pronounced inhibitory effect on iodine accumulation and doses of 5.0 Mg. completely inhibited the uptake for nearly twenty-four hours (Fig. 1). In Table 1 are shown the various grades of inhibition induced by different doses of this compound in comparison with various doses of thiouracil, methylthiouracil, propylthiouracil, and 2-mercaptoimidazole. From these data it was estimated that the activity of 1-methyl-2-mercaptoimidazole was approximately 100 times that of thiouracil.

Preliminary studies on thirty patients with hyperthyroidism treated with this compound have confirmed a high degree of effectiveness.

ACKNOWLEDGMENTS

The 2-mercaptoimidazole was generously supplied by Dr. R. O. Roblin and associates of the Stamford Research Laboratories of the American Cyanamid Company. We are indebted to Dr. D. C. Hines of the Eli Lilly Research Laboratories for the 1-methyl-2-mercaptoimidazole used in these studies.

MALCOLM M. STANLEY AND E. B. ASTWOOD
From the Joseph H. Pratt Diagnostic Hospital and the Department of Medicine, Tufts Medical School,
Boston, Massachusetts

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SAMPLE ABSTRACTS

BEATH, O. A., C. S. GILBERT, and H. F. EPPSON. (Wyoming Agric. Expt. Sta., Laramie.) / The use of indicator plants in locating seleniferous areas in western United States. II. Correlation studies by states. / Amer. Jour. Bot. / 26(5): 296-315. 1939.—Studies were made of the Se content of 563 specimens of native plants from 12 of the western states, correlating the presence of Se in plants with the geological formation in which the plants grew. Special attention was given to spp. of Stanleya, Oonopsis, Xylorrhiza and Astragalus as primary indicators of Se in formations contemporaneous with known seleniferous formations of Wyoming and also in formations not previously investigated. The Stanleya and Astragalus spp., because of their wide distribution, proved especially valuable as indicators. The following are added to the list of known seleniferous formations: Pennsylvania limestones of southeast and central Nevada; Payette Lake sediments in southwest Idaho; Carbonaceous and limy shales in Provo Canyon, Utah; Tertiary sediments in Tonto Basin, Arizona; White Tank Monzonite near Twenty-nine Palms, California; the Monkopi formation in southern Utah and northern Arizona; and certain areas in southeast Idaho, northwest Utah, southeast Oregon and Nevada.—O. A. Beath.

DeSMIDT, F. P. G. (Med. Res. Lab., Kenya.) / Rapid production of autogenous vaccine for treatment of pneumonia. / Brit. Med. Jour. / 1938(4065): 1140-1142. 1938.—An autogenous vaccine made directly from mouse peritoneal washings ("M. P. W." vaccine) can be ready in less than 12 hours, as compared with several days required by the usual method for preliminary typing, growth, isolation of colonies and subsequent sterility tests. DeSmidt trusts to the dominance of the type of pneumococcus inj. into the mouse, to the disappearance in the mouse's body of other bacteria which may have been in the sputum, and to strictly aseptic technic throughout to secure pure, sterile vaccine. His procedure is as follows: Inoculate 2 mice intraperitoneally with 0.5 ml. of rather thick saline sputum emulsion; chloroform the mice when moribund—do not allow them to die; take utmost precautions for sterility of board, tools, skin and abdominal wall before opening peritoneal cavity; Gram-stain a smear of peritoneal exudate for verification; make a preliminary washing of peritoneal cavity into 1 ml. of plain saline for bile solubility and agglutination tests (this preliminary wash may be omitted), make peritoneal washing in 4 ml. of 0.5% phenol saline into sterile centrifuge tube with sterile stopper; centrifuge at 6000 r. p. m. for 30 sec., pipette supernatant pneumococcus susp. into sterile cotton-plugged tube; cover with rubber cap; immerse in water-bath at 60° for 30 min.; place the killed susp. in vaccine bottle. The Burroughs Wellcome opacity tube is used to estimate the strength of suspensions, usually 1,000 to 3,000 millions per ml. Dose 1,000 millions of cocci in 0.5 ml. The vaccine may be administered immediately after bottling, the usual sterility tests being dispensed with—J. B. Paton.

STEFANELLI, A. (U. Rome.) / Il metabolismo dell'uovo e dell'embrione studiato negli anfibi anuri. II. L'assunzione di ossigeno. [Metabolism studies on amphibian (anuran) eggs and embryos. II. The oxygen intake.] / Arch. Sci. Biol. [Naples] / 24: 411-441. 1938.—By a microrespirometric method the O_2 uptake of individual eggs of Bufo vulgaris and B. viridis was detd. before and after fertilization (which was carried out in the manometer), gastrulation, and neurulation. The O_2 consumption increased during gastrulation and after completion of the neurulation; after that it ran proportional to the number of cells until absorption of the yolk was complete. Equal wts. of eggs, regardless of individual egg size, take up equal amts. of O_2 during equal periods of time or development. The faster developing B. viridis consumes more O_2 per unit of time, but the same amt. as B. vulgaris for the same degree of development.—H. Sobotka.

ECKSTEIN, K. (Forstliche Hochschule, Eberswalde.) / Die "Abbrüche" des Waldgärtners, Myelophilus piniperda L. / Fortwiss. Centralbl. / 61(2): 33-42; (3): 81-92. 4 fig. 1939.—The larva bores into pine twigs and hollows them out so that they eventually break off. 50 to 150 twigs may be lost by a single tree during a season. Similar damage is done by M. minor and Pityophthorus glabratus.—W. N. Sparhawk.

POĬUROVSKIĬ, S. D. (Inst. Usovershenstvovaniia Vrachei, Leningrad.) / [Photoelectrometric determination of protein fractions of blood serum.] / [In Russ. with Ger. summ.] Biokhimiia / 3(6): 713-722. 1938.—Photoelectric detn. of blood serum proteins can be conducted on samples as small as 0.1 ml. with an error of 2% or less. In a sample diluted 1:100, the globulin fraction is detd. first by half-saturation with $(NH_4)_2SO_4$ and comparing the light absorption of the suspension with that of a prepared blank. The sample is filtered and the albumin fraction in the filtrate detd. by addition of 5% sulfosalicylic acid and again comparing with a blank. The readings are converted to percentages by means of tables.—E. K. Johnson.

MENKIN, VALY. (Harvard U.) / Études sur l'inflammation. / Rev. Immunol. [Paris] / 5(2): 113-137. 1939.—In discussing the mechanism of the inflammatory reaction and its rôle in immunity, the author reviews his many previous studies. The inflammatory process tends to localize and ultimately to destroy the irritating factor. This leads to repair. The exudation of plasma, detd. by a rise in intracapillary pressure and an increase in capillary permeability, is due to a crystalline, non-protein, diffusible substance liberated by the injured tissues. The so-called "leukotaxine" is not histamine, but seems to be a polypeptide. It is thought to be responsible also for the migration of leucocytes to the inflamed region. As the inflammatory process progresses, lymphatic drainage is blocked by thrombi in the lymphatic channels and coagulated plasma in the injured site. The fixing capacity of an inflammation is proportional to the injury exerted by the irritant. The irritants that cause necrosis produce a rapid local reaction which neutralizes their power of dissemination. The contrary is true of weak irritants. This explains the action of the pyogenic bacteria, their rapid invasion and the rôle of the inflammatory reaction in the development of immunity and host resistance. The walling off of the inflamed area tends to produce a local acidity that determines the cytology of the region. Polymorphonuclear leucocytes persist in an exudate whose pH is above 7; if the pH falls to 6.8 or 6.9, mononuclears predominate; in an exudate with pH of 6.5 or less, all types of cells are destroyed.—W. I. Kelly.

CURTIS, JOHN T. (U. Wisconsin.) / The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. / Amer. Jour. of Bot. / 26(6): 390-399. 9 fig. 1939.—Ten spp. of Rhizoctonia were isolated from 23 spp. of orchids from various habitats in the U. S. and Central America. Two new species and a new var. are descr.: R. borealis, from Goodyera repens var. ophioides and Spiranthes gracilis, Wisconsin; R. subtilis var. nigra, from S. cernua, Wisconsin; and R. moniliodes, from Habenaria dilatata, H. hyperborea, Liparis liliifolia, Pogonia ophioglossoides and S. romanzoffiana, Wisconsin, and Sobralia pleiantha, Costa Rica. No evidence of specificity was found, as one orchid species could harbor several fungus spp. and any one fungus sp. could attack several orchid spp. The fungi were correlated with ecological habitat rather than with orchid spp. Symbiotic germination tests, using fungi isolated from a given orchid in combination with the seeds of that orchid, indicated in most cases that the fungi were unable to induce seed germination. The symbiotic relationship is considered to be one of parasite and host, with the orchid deriving no benefit from the fungus in its roots.—J. T. Curtis.

Abbreviations

alpha	α	distribution	distr.	lethal dose, minimum lethal dose	M.L.D.	percent	%
amount	amt.	experiment(al)	expt., exptl.	male, males	♂, ♂♂	pound(s)	lb., lbs.
approximately	approx.	female, females	♀, ♀♀	microgram(s)	γ	second(s)	sec.
beta	β	foot, feet	ft.	milligram(s)	mg.	solution	soln.
concentration	conc.	gamma	γ	milliliter	ml.	species	sp., spp.
cubic centimeter	ml.	gram(s)	g.	minute(s)	min.	temperature	temp.
described	descr.	hour(s)	hr., hrs.	ounce(s)	oz.	ultra-violet	u.-v.
determined	detd.	inch(es)	in.			variety(ies)	var., vars.
						volume(s)	vol.

Use chemical symbols for the elements and the simpler inorganic compounds.

NEW BOOKS

ANNUAL REVIEW OF PHYSIOLOGY, Volume XI, 1949, ix+643 pp. Annual Reviews, Inc. Stanford, California. $6.00.

The present volume begins the second decade of life for the Annual Reviews of Physiology. As compared with human chronology, it might therefore be regarded as pre-adolescent, a period of great stress and strain, and characterized by rapid growth and awkward locomotion. The analogy is perhaps not too far-fetched, since the Editors in their preface note that the current reports in physiology are increasing in geometric progression and that their task in preparing the annual volume is one requiring continuous adaptation. One can sympathize with them in their labors, the more so since the volume defies reviewing by any of the ordinary artifices. For how can one review a volume written by twenty-two different authors, covering the entire field of physiology, condensed to a degree that each sentence summarizes itself an entire article or series of articles, and even then compressed still further so that one author plaintively remarks that he read 1000 articles only to end by reviewing 288 of them?

The task, then, of preparing such a volume is formidable, and it is impossible to compare its form, substance, literary quality and scientific validity with those properties of similar books. One might question whether the effort is worth the trouble. Indeed, considering the amount of condensation represented in such a volume, one can ask whether it performs any significant function not provided by the Index and Abstract periodicals. There seems to be danger that the Annual Reviews may become merely a list of the articles published during the year, for the reviewers, more and more in recent times, are falling into what might be called the Annual Reviews style, in which each sentence begins: "Doe and Everyman (1948) found that . . . ." The Editors are obviously aware of this tendency and remark that they have "earnestly pleaded with our reviewers to consider only those papers which they deem particularly noteworthy contributions to our science and to reduce coverage of other papers to mere listing."

It seems ridiculous for productive investigators to spend the time necessary for mere listing of unimportant papers. Such lists are available, and better done, in the Index journals. It also seems unwise to restrict coverage to the papers published in a single year, for by so doing concepts are taken out of historical context. It moreover seems unprofitable for a reviewer, striving for completeness, to use space and energy on topics not directly related to his own interests and experience. On the contrary, a better policy would be to write with the greatest possible insight on a few topics, indicating their historical development, a critical analysis of their present status and a projection into the future of their probable development. Such reviews are the proper concern of investigators. They represent the thought out of which progress is made. No apology need be made for lack of completeness—in a decade each field would be considered by ten of its leading exponents. If the thoughts of its leaders do not define the subject material of a field, what does?

NATURAL PRODUCTS RELATED TO PHENANTHRENE. 3rd Edition, 1949. Louis F. Fieser and Mary Fieser. American Chemical Society Monograph No. 70. Reinhold Publishing Corporation, 330 W. 42nd St., New York, N. Y. xii+704 pp. $10.00.

The continuing interest in the chemistry of the steroids has led to a vast amount of work since the 2nd edition of this book was published in 1937. The present edition is, therefore, more nearly a new book than an ordinary revision. Appropriate recognition of stereochemical information has been accomplished by the addition of a special chapter by Dr. Richard B. Turner. The large amount of literature relating to partial and total syntheses has been carefully summarized. Considerable attention has been devoted to new methods for characterizing steroid compounds, including optical rotation data, infrared and ultraviolet absorption spectra and x-ray diffraction. Last but not least, the systematic consideration of the field has compelled the authors to come to grips with the nomenclature problem. The result is a new systematic nomenclature which departs from the older schemes only where consistency demands it.

Quite aside from the great virtues of this book as a summary of purely chemical information, it is of great importance to physiological endocrinology. Indeed, it is monolithic in character, for were no other reference available, it would provide an astonishingly complete account of the known information about the steroid-secreting endocrine glands. Not the least of its accomplishments is the avoidance of questionable data and controversial interpretations regarding the regulation of endocrine organs. Fact and fancy are so promiscuously intermixed in the literature of reproductive and adrenal physiology that it takes rare ability to cleave through the claims and counter claims and arrive at a balanced estimate of the total situation.

A word, at least, should be said to compliment the lucid, unobtrusive and generally excellent literary style of the authors. The book is nicely printed with a legible typeface on good quality paper. Profuse use has been made of tabular material and of structural formulas. Documentation is extensive, the references being set as footnotes for the convenience of the reader. The volume is completed by adequate and accurate author and subject indexes. It is a pleasure to commend this admirable monograph to the attention of everyone interested in endocrinology.

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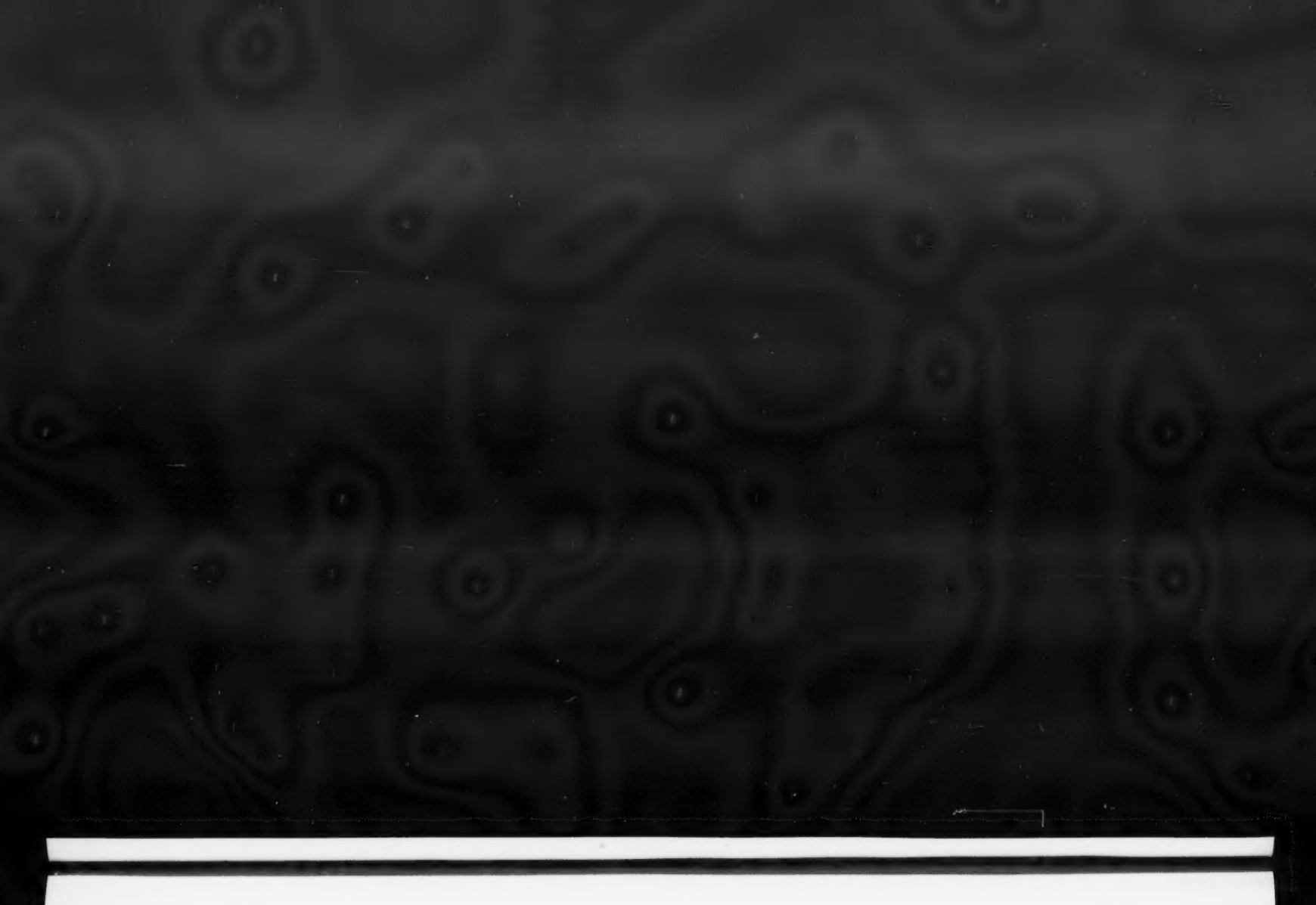
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